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**Characterisation of the naturally-acquired  
antibody response to the surface of *Plasmodium*  
*falciparum* infected erythrocytes**

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**This thesis is dedicated to my parents**

**John and Ursula**

**with love and thanks**





# Abstract

The surface of *P. falciparum*-infected erythrocytes is modified by the insertion of immunogenic parasite-encoded proteins; the most extensively studied family is *Plasmodium falciparum* erythrocyte membrane protein 1, (*PfEMP1*). *PfEMP1* undergoes clonal antigenic variation and is responsible for mediating cytoadherence of infected erythrocytes within the microvasculature, characteristics underpinning the virulence associated with *Plasmodium falciparum* infection. Protection against disease following exposure to a parasite displaying a particular variant of *PfEMP1* is associated with agglutinating antibodies against that variant, antibodies associated with protection. However, the role of cross-reactive responses to heterologous isolates in protection from subsequent disease remains poorly understood. The target and epitope specificity of both the variant specific protective response and any cross-reactive antibody response is unknown.

Individuals from two areas with differing transmission characteristics were screened at the end of a low transmission season for recognition of a panel of laboratory and clinical isolates. Using in particular an isolate selected to express one specific variant of *PfEMP1*, A4 *PfEMP1*, on the surface of the host erythrocyte, the relationship between the presence of asymptomatic parasite carriage and cross-reactive antibody responses was established. Subsequently the domain and interdomain regions of A4 *PfEMP1* were cloned and expressed as recombinant proteins and used to screen the same individuals in an attempt to identify the targets for these measured responses. This study demonstrated differences in antibody acquisition between domains, individuals and areas. In a longitudinal study, the

interaction between having detectable parasites and the ability to recognise the surface of erythrocytes infected with four different parasite isolates led to the identification of a susceptible group of children responsible for the burden of clinical malaria in this area.

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# Chapter 1

## Introduction

### *Plasmodium falciparum* malaria – a perspective

In 2002, 2.2 billion individuals were at risk of *Plasmodium falciparum* malaria and there were approximately 515 million episodes of clinical malaria caused by this parasite worldwide (Snow, Guerra et al. 2005). 70% of all *P. falciparum* malaria cases occurred in sub-Saharan Africa with 25% occurring in South East Asia. It is estimated that 1 million people die in Africa alone as a direct result of *P. falciparum* malaria each year with the majority of these being less than 5 years of age (Snow, Craig et al. 1999). In areas of stable endemic transmission around 25% of all mortality in those aged less than 5 years has been attributed directly to malaria (Snow, Craig et al. 2003). Additionally, many randomised controlled intervention trials aimed at reducing the incidence of infection have resulted in reductions in mortality in excess of that attributed to malaria alone (Molineaux 1997; Snow, Korenromp et al. 2004). This has led to speculation that not only does *P. falciparum* infection directly cause death in some individuals but in many others it is a contributor. Despite a continued decline in all-cause mortality in children less than 5 years in sub-Saharan Africa since the 1960s, a similar downward trend in malaria-specific mortality has reversed over the last 15 years (Snow, Trape et al. 2001). The reasons for this are undoubtedly complex but the most parsimonious explanation is unquestionably the rapid decline in efficacy of chloroquine, a cheap, effective and readily available anti-malarial which, until the mid 1990's was in widespread use throughout sub-Saharan Africa.

Widespread implementation of successful and accessible case-management of disease using effective anti-malarial drugs, such as the artemisin-based combination treatments, and the use of preventative measures such as insecticide-treated bednets, capable of reducing all childhood deaths by around 20% (Lengeler 2004), may reverse the rising mortality due to malaria in Africa. However, this will require a far greater financial commitment from the Governments of wealthy countries than currently exists. While these strategies could be implemented to great effect today, it is the development of an efficacious malaria vaccine that remains the holy grail of basic malaria research. Intense research efforts towards this goal have been undertaken over the past 20 years, however the complex biology of the malaria parasite has resulted in an effective vaccine remaining elusive. More recently, a renewed global commitment to funding malaria vaccine research, (Malaria Vaccine Initiative MVI, Global alliance for Vaccines and Immunisations GAVI)(Boseley and Elliott 2005) and the rapid development of new technology allowing fuller access to the parasite genome have led to a new and determined effort to develop an effective and accessible malaria vaccine. The studies undertaken towards this thesis aim to improve our understanding of the naturally occurring acquired immune response to the asexual intra-erythrocytic stage of the *P. falciparum* life cycle and thus in turn aid progress towards the development of a vaccine effective against *P. falciparum* malaria, .

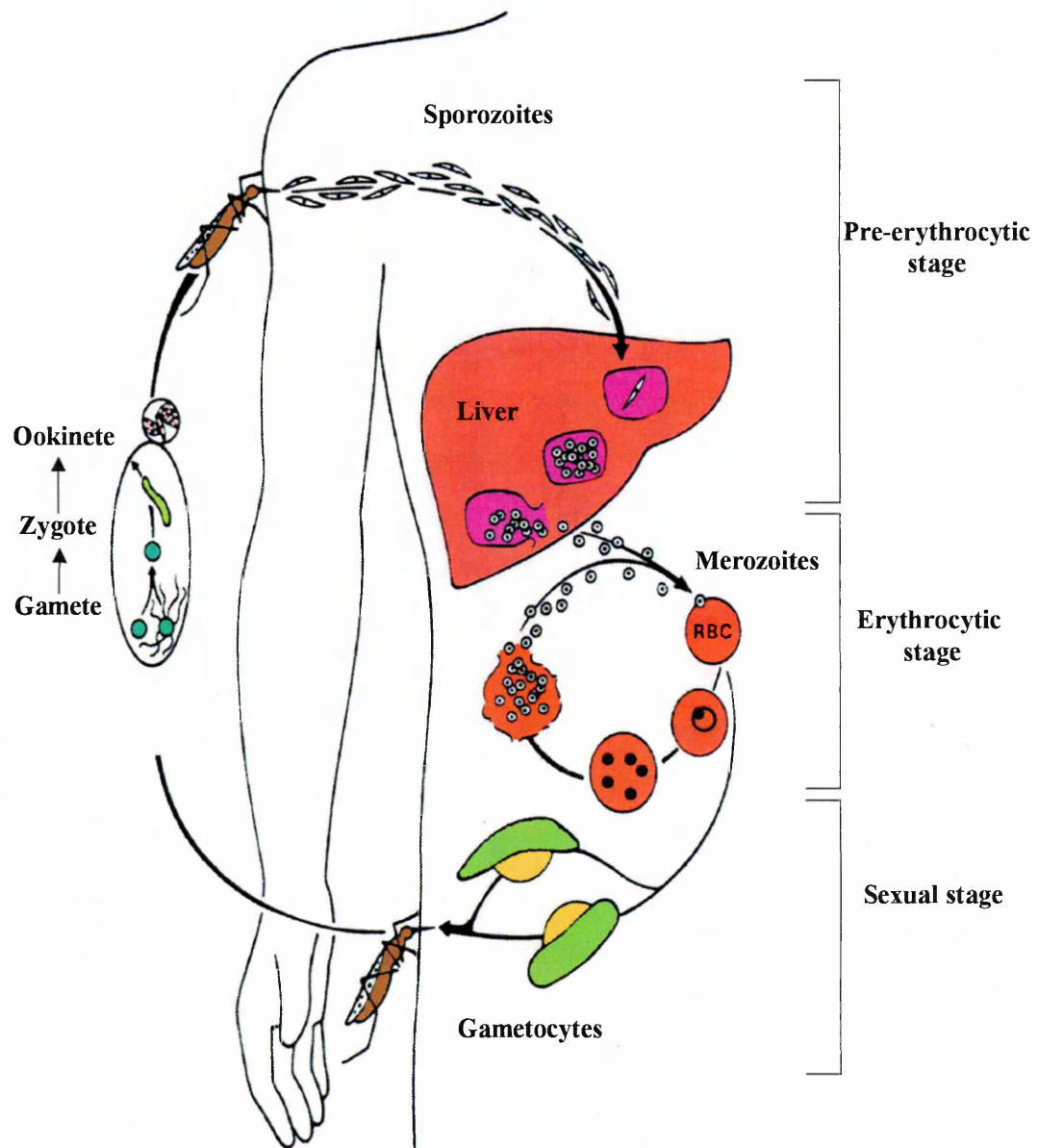
### **Plasmodium life cycle (Figure 1.1)**

The infected female anopholene mosquito injects between 1 and 100 infective sporozoites from her salivary glands into the host bloodstream at the time of blood



meal (Rosenberg, Wirtz et al. 1990; Ponnudurai, Lensen et al. 1991). These rapidly pass through the bloodstream to the liver where 5-10% successfully invade hepatocytes (Ferreira, Enea et al. 1986). Within the hepatocyte these sporozoites divide forming pre-erythrocytic schizonts in which around 30,000 daughter merozoites develop. After a minimum of 5 ½ days, the merozoites lyse the hepatocyte and enter the bloodstream within the hepatic sinusoids. The stage of development from sporozoite inoculation to hepatocyte rupture is the pre-erythrocytic stage of infection and, other than a local inflammatory reaction at the site of inoculation, is asymptomatic to the host.

The erythrocytic-stage of infection begins with the entry of merozoites into host erythrocytes. After invasion they divide to become schizonts containing around 16-32 merozoites. The erythrocyte eventually ruptures releasing these daughter merozoites into the bloodstream where they each invade a fresh erythrocyte and begin the cycle again. The entire erythrocyte stage of infection takes around 48 hours from invasion to rupture of the erythrocyte and this leads to an exponential increase in parasites. It is thought that it is the intermittent release of merozoites which give rise to the symptoms of malaria in the host through stimulation of the release of endogenous pyrogens, which in turn are responsible for the classical cyclical fevers. A proportion of merozoites enter the sexual stage by differentiating into male and female gametocytes within erythrocytes. These sexual forms are responsible for transmission of *P. falciparum* through ingestion by feeding mosquitoes. Once ingested, they travel to the mosquito's midgut, where they mature and fuse to form zygotes. Zygotes develop into ookinetes, which invade midgut



**Figure 1.1**

The life cycle of *Plasmodium falciparum*, indicating the three stages occurring within the human host.

epithelial cells and develop into thousands of sporozoites. These enter the mosquito salivary glands from whence they transmit a further *P. falciparum* infection when the mosquito feeds again on a human host. This thesis will only discuss humoral immune responses to antigens present during the intra-erythrocytic stage of the life cycle.

### **The global distribution of malaria**

The probability of malaria transmission occurring is dependent upon climatic and ecological factors. Conditions appropriate for the survival of the anopheline mosquito, the major vector transmitting *P. falciparum*, and for the development of sporozoites within the vector include at least 80mm of rainfall annually and average temperatures greater than 18°C for at least five contiguous months of the year (Craig, Snow et al. 1999). Even with these climatic necessities, transmission can be influenced by human activities such as agriculture, urbanisation, mass population movement and malaria control programmes.

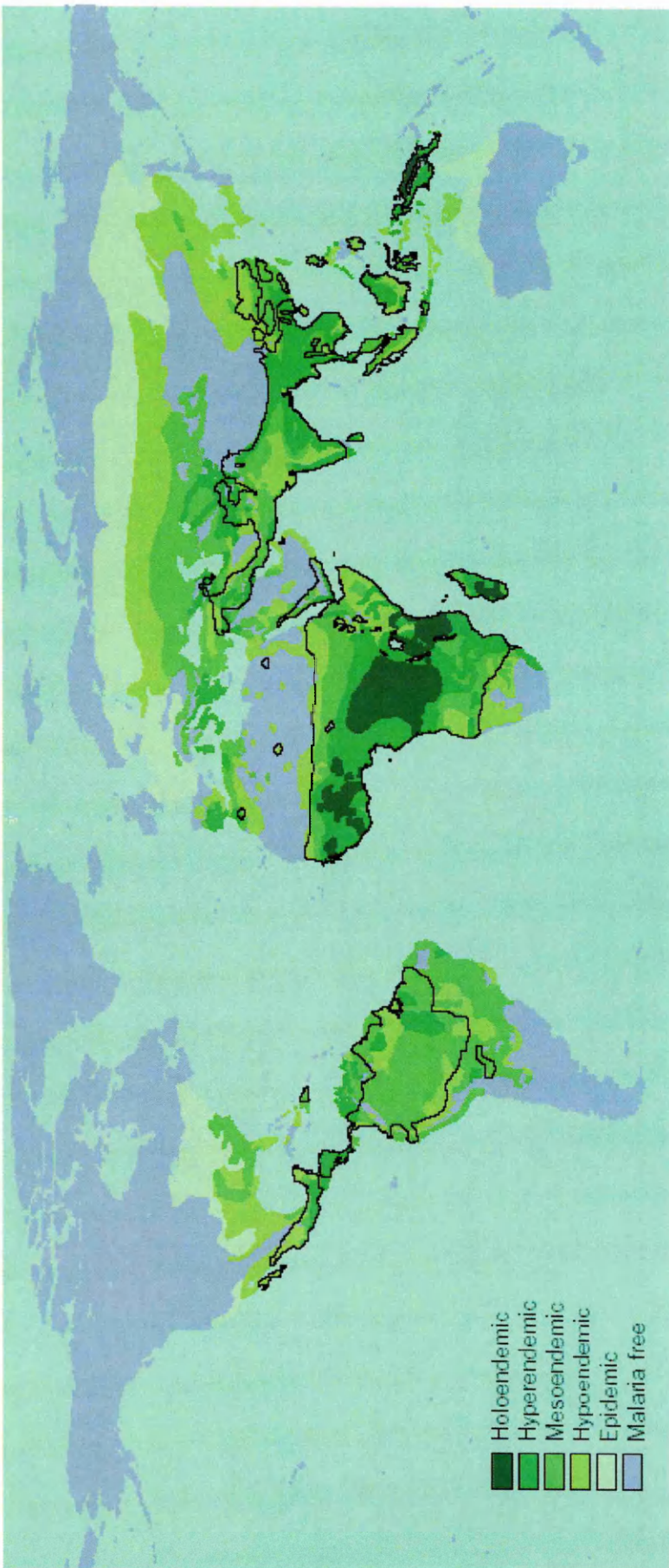
Until recently, the only global map of malaria endemicity dated from 1968 (Lysenko and Semashko 1968). This map combined historical records and maps of different malariometric indices collected up until the late 1960's. Efforts to update this, taking into account both population denominator changes and malaria control efforts over the last 30 years have demonstrated that despite the total land area supporting malaria being reduced by half, there has been a 2 billion increase in the total population exposed to malaria (figure 1.2) (Hay, Guerra et al. 2004; Snow, Guerra et al. 2005).

## **Measuring malaria transmission**

Malaria is most commonly classified according to whether transmission is stable or unstable. Rather than two completely discrete situations, individuals reside in areas across a spectrum of risk encompassing both classifications. Under stable endemic conditions, the prevalence of infection is relatively high and persistent. Transmission remains relatively insensitive to environmental changes and there is little year-to-year variability although seasonal fluctuations can occur. Unstable areas conversely are characterised by great variability in transmission over time.

A number of measures have been used to estimate the intensity of transmission. A widely used measure is the prevalence of peripheral blood stage infections among a community, or the parasite rate or more accurately parasite ratio. Parasite ratios do not provide a direct quantitative estimate of new infections unless the survey is restricted to infants, in which case the age-related acquisition of parasitaemia can give a direct measure of transmission, they can vary widely from season to season within the same year and variation over small distances has been recorded (Smith, Charlwood et al. 1995). Nonetheless, the parasite ratio does give a measure of intensity of transmission and has thus been used to define endemicity in classical malaria epidemiology. Both parasite and spleen ratios (the proportion of a population that exhibit splenomegaly) were used to define classes of endemicity as outlined in table 1.1 (Metselaar and Van Theil 1959). Although widely used this classification creates artificial groups from a natural continuous spectrum and hides important differences between localities, for example an area where an individual

may experience five infective bites per year would be classified in the same bracket as an area where an individual may receive five hundred infective bites in a year.



**Figure 1.2 The Lysenko map of malaria endemicity (Lysenko and Semashko 1968)**

The black line represents the 2002 estimate of the geographical limit of malaria risk. (Adapted from (Hay, Guerra et al. 2004))

Using malariometric indices such as parasite rate in endemic areas may imply that this can give us some information about malaria illness and death. However this is not straightforward. During the critical period when children are most at risk of severe disease, they spend most of their time asymptotically parasitised. In fact the prevalence of parasitaemia continues to rise during the period when the incidence of clinical malaria and the risk of death falls, (figure 1.3) (Marsh, Otoo et al. 1989). Parasite rates reach saturation and remain at this level throughout older childhood and adolescence and only begin to fall again in adulthood. The level at which saturation occurs correlates with transmission intensity, as measured by the entomological inoculation rate (EIR), although even at low transmission levels, the prevalence at which parasite rates saturate is high (Beier, Killeen et al. 1999). Saturation is maintained by chronic infection and re-infection. Although in areas of high transmission, a greater proportion of the population is parasitised at any one point, this does not reflect the incidence of clinical disease in a straightforward manner. Indeed, a study investigating the burden of severe malaria in five sites with differing transmission intensities demonstrated that population rates for severe malaria at first rose with increasing transmission intensity, then reached a plateau after which, at very high levels of transmission they fell (Snow, Omumbo et al. 1997). A finding subsequently reproduced by others (Modiano, Sirima et al. 1999) (Reyburn, Mbatia et al. 2005).

The EIR is a measure of the number of infective bites an individual receives over a particular unit of time. Usually the EIR is expressed per year in order to take into account the seasonality of transmission in many areas. The EIR is subject to many

Type	Spleen rates	Parasite rates	Description
Hypoendemicity	Not exceeding 10% in children aged 2-9 years	Not exceeding 10% in children aged 2-9 years but may be higher for part of the year	Areas where there is little transmission
Mesoendemicity	Between 11 and 50% in children aged 2-9 years	Between 11 and 50% in children aged 2-9 years	Typically found in rural communities in sub-tropical zones where wide variations in transmission risk exist
Hyperendemicity	Constantly over 50% in children aged 2-9 years; also high in adults (over 25%)	Constantly over 50% in children aged 2-9 years	Areas where transmission is intense but seasonal.
Holoendemicity	Constantly over 75% in children aged 2-9 years: low in adults	Constantly over 75% in infants aged 0-11 months	Perennial, intense transmission

**Table 1.1**

**Classification of endemicity (Metselaar and Van Theil 1959)**

Adapted from (Snow and Gilles 2002)



ecological and climatic constraints and varies widely across sub-Saharan Africa both in space and time (Hay, Rogers et al. 2000).

### **The relationship between transmission, age and distribution of disease**

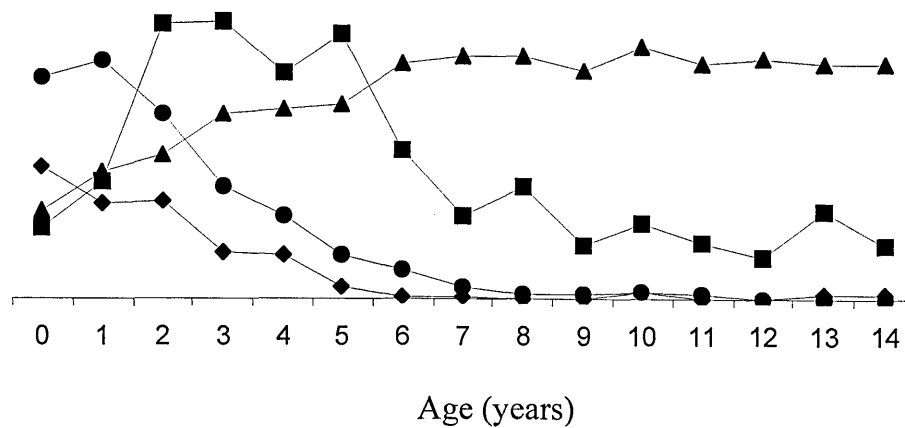
In non-immune individuals of any age, infection with *P. falciparum* parasites will almost always result in clinical disease. In stable endemic areas acquired immunity results in young children bearing the brunt of the morbidity and mortality. Up until the age of 6 months, infection and clinical disease are rare, producing only mild symptoms, and parasitaemias are usually low (Snow, Nahlen et al. 1998). This resistance is a result of passive transfer of maternal antibodies, persistence of haemoglobin F and other factors such as avoidance of vectors by the mother (Sehgal, Siddiqui et al. 1989; Hogh, Marbiah et al. 1995). From around 6 months of age, children become susceptible to severe disease and death. Under high transmission pressure, disease incidence peaks in the first year of life and as a result of the development of functional immunity, by year four children have few clinical episodes and these are usually mild (Snow, Omumbo et al. 1997; Bloland, Boriga et al. 1999; Reyburn, Mbatia et al. 2005). In areas with lower transmission characteristics the disease-incidence curve is flattened and the peak occurs at a later age, (figure 1.3)(Snow, Bastos de Azevedo et al. 1994; Snow, Omumbo et al. 1997; Modiano, Sirima et al. 1999; Rogier, Tall et al. 1999; Reyburn, Mbatia et al. 2005). Clinical manifestations of severe malarial disease differ with age, with severe malarial anaemia occurring at a consistently younger age across the transmission spectrum (Snow, Bastos de Azevedo et al. 1994; Marsh, Forster et al. 1995; Snow, Omumbo et al. 1997; Reyburn, Mbatia et al. 2005). It is not surprising then that in

areas of high transmission severe malarial anaemia dominates the spectrum of disease with cerebral malaria becoming increasingly more important as transmission levels drop.

### **Clinical spectrum of disease**

*P. falciparum* malaria causes a spectrum of clinical presentations from asymptomatic parasitisation, through a mild febrile illness to severe disease and death. The outcome of any sporozoite inoculation is dependant upon a multitude of factors including host genetic susceptibility, host acquired immunity, host nutrition, parasite virulence factors and access to prompt and effective treatment. It is estimated that between 0.1 and 0.5% of all malarial infections result in severe life-threatening disease (Greenwood, Marsh et al. 1991). Although a small proportion, the fact that the overall numbers of clinical attacks is so large, this results in a high number of cases of life-threatening infection (Snow, Guerra et al. 2005) (Figure 1.4).

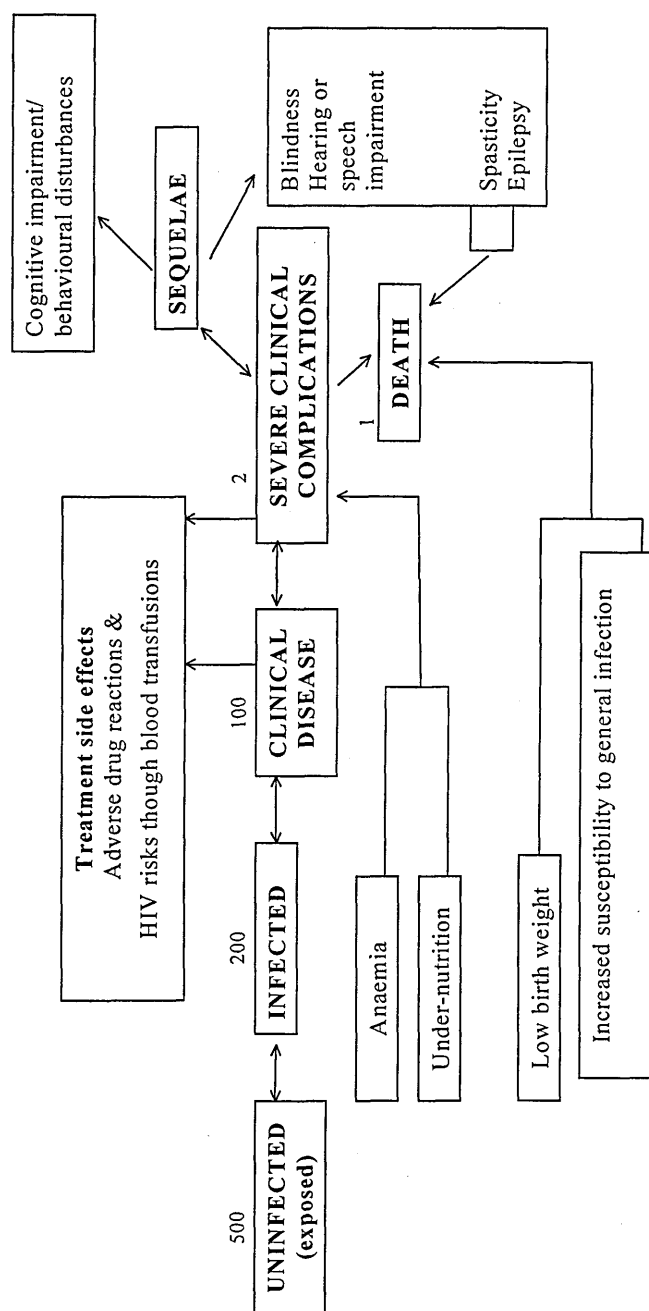
The first clinical symptoms of infection with *P. falciparum* are non-specific. In common with many infectious diseases, high fever, muscle aches and headaches predominate. Specific to synchronised experimental malaria infections is the characteristic cyclical nature of the fevers, recurring every 48 to 72 hours coinciding with the erythrocytic growth phase of the life cycle and eventual erythrocyte rupture; in nature, individuals seldom exhibit such tightly synchronous infections (Kwiatkowski and Greenwood 1989). In those individuals resident in malaria endemic areas, over time, frequent exposure results in the development of non-sterile acquired immunity that protects against moderate to severe disease but not infection *per se*. In infants, young children and pregnant women in endemic areas, non-



**Figure 1.3**

Relative risks of infection (triangles), morbidity (squares), severe disease (circles) and death (diamonds) among a population aged 0-15 years located in a stable endemic area on the Kenyan coast.

(Adapted from (Snow and Gilles 2002))



**Figure 1.4**

# Public Health burden of malaria

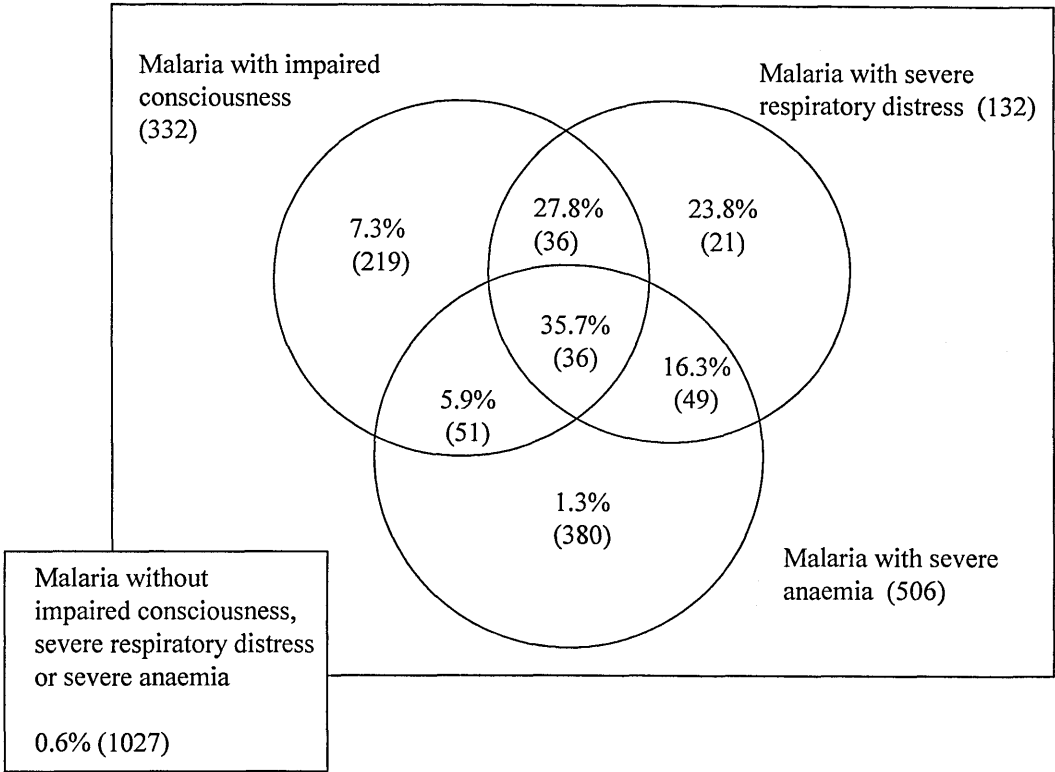
The numbers are to give a guide to the proportion of individuals suffering from each endpoint only. They are based on averages from many studies. (Adapted from (Marsh 1992; Snow and Gilles 2002))

exposed travellers to endemic areas, those resident in unstable epidemic-prone areas and those with impaired immunity, malaria infection can result in severe disease and possibly death.

Severe malaria in African children has been classified into three overlapping syndromes: cerebral malaria, severe anaemia and respiratory distress (Marsh, Forster et al. 1995)(Figure 1.5).

Cerebral malaria is characterised by impaired consciousness with or without seizures. The mortality rate varies from 15-33% in different settings, although comparison can be made difficult by variability in the definition of cerebral malaria (White, Warrell et al. 1985; Molyneux, Taylor et al. 1989; Kwiatkowski, Molyneux et al. 1993; Marsh, Forster et al. 1995). Outcome is associated with depth of coma, (Taylor, Molyneux et al. 1988; Molyneux, Taylor et al. 1989; Marsh, Forster et al. 1995), but for those who recover the return to consciousness can be rapid (Turner 1997). Approximately 11% of those who survive cerebral malaria have neurological sequelae (Newton and Krishna 1998). Factors associated with sequelae include protracted seizures, prolonged and deep coma and hypoglycaemia (Bondi 1992; Walker, Salako et al. 1992; van Hensbroek, Palmer et al. 1997) as well as the development of raised intracranial pressure (Newton, Crawley et al. 1997) As not all cerebral malaria patients suffer neurological sequelae, it must be that in the majority of cases the pathological processes underlying coma do not involve extensive irreversible destruction of the host's cells

Severe malaria anaemia (SMA) is defined as haemoglobin of 5g/dl or less in the presence of *P. falciparum* parasites with or without respiratory distress (WHO 2000). Around 25% of all malaria admissions met these criteria in a stable endemic



**Figure 1.5**

Total numbers are given in parenthesis and mortality is given as a percentage.  
(Adapted from (Marsh, Forster et al. 1995))

area on the coast of Kenya (Marsh, Forster et al. 1995). Those children who were otherwise well did considerably better than those children with co-existent respiratory distress, with a mortality rate of only 1.3% compared to 16.3%, (figure 1.5). Traditionally respiratory distress had been thought to be a manifestation of cardiac failure, reviewed in (English 2000). However, more recently studies have highlighted the contribution to respiratory distress of lactic acidosis and hypoperfusion secondary to both hypovolaemia and poorly deformable erythrocytes (English, Waruiru et al. 1997; Dondorp, Kager et al. 2000; Maitland, Levin et al. 2003; Maitland, Pamba et al. 2003). It is clear from figure 1.5 that the burden of mortality within severe malaria as a syndrome falls with those children displaying respiratory distress either alone or in combination with severe anaemia or impaired consciousness.

Other prognostic indicators of a poor outcome in severe malaria include pulmonary oedema (Deaton 1970; Marsh, Forster et al. 1995), hypoglycaemia (Taylor, Molyneux et al. 1988; Molyneux, Taylor et al. 1989; Marsh, Forster et al. 1995), cerebral oedema (Newton, Kirkham et al. 1991; Newton, Crawley et al. 1997) and renal failure (Warrell 1989)

### **Immunology of malaria**

The well-described age-related protection against severe disease and death due to *P. falciparum* malaria in stable endemic areas is a result of acquired malaria-specific immunity. This is not to say innate mechanisms of immunity do not play an important additional role. Even in those individuals exposed to malaria for the first time, it is not necessarily the case that severe disease will be the eventual outcome. Rather a spectrum of resolutions is possible from death at one extreme to those who

remain resistant even to mild clinical disease and infection. This spectrum is a result of innate mechanisms of protection and these include genetic polymorphisms affecting the ability of the parasite to infect and or replicate in host cells the immune response and molecules involved in pathogenesis.

### **Genetic resistance to malaria**

When discussing genetic mechanisms of resistance to malarial disease of particular note are conditions affecting the structure of the  $\beta$ -globin chain of haemoglobin such as sickle cell trait (HbAS), haemoglobin C (HbC) and haemoglobin E (HbE), conditions affecting the rate of synthesis of globin chains such as the thalassaemia syndromes, the level of glucose-6-phosphate dehydrogenase (G-6PD) as well as abnormalities of the red cell cytoskeleton such as ovalocytosis. A summary of evidence illustrating the effect of the haemoglobinopathies on incidence of clinical malaria is given in table 1.2.



Trait	Study	Protection	Reference(s)
HbAS	Cross-sectional survey	Reduced parasitaemia Reduced malaria-specific mortality	(Allison 1954)
	Case-control studies	90% protection against severe malaria (cerebral malaria and severe anaemia) 60% protection against clinical malaria leading to hospital admission	(Willcox, Bjorkman et al. 1983; Hill, Allsopp et al. 1991; Marsh 1992)
	Cohort study	55% protection from all-cause mortality between 2 16 months of age (high malaria transmission area) Reduced risk of high density parasitaemia 50% protection from mild malaria, 75% protection against admission to hospital with malaria and 90% protection against severe malaria disease	(Aidoo, Terlouw et al. 2002)  (Williams, Mwangi et al. 2005)
$\alpha$ -Thalassaemia	Micro-epidemiological studies	Gene frequencies low in non-malarious areas Elsewhere, proportional to historical incidence of malaria	(Flint, Hill et al. 1986; Yenchitsomannu Summers et al. 1986)
	Case-control studies	60% protection from severe malaria for homozygotes and 34% for heterozygotes Increased incidence of mild malaria	(Allen, O'Donnell et al. 1997)  (Oppenheimer, Hill et al. 1987; Willia Maitland et al. 1996)
		Prevalence of homozygotes and heterozygotes significantly less amongst severe malaria cases and malaria-attributable deaths	(Williams, Wambua et al. 2005)
$\beta$ -Thalassaemia	Micro-epidemiological Studies	A decline in the population frequency that correlate with altitude and historical incidence of malaria	(Hill, Bowden et al. 1988)
	Case-control study	Protection from hospital admission with malaria of 50%	(Willcox, Bjorkman et al. 1983)
HbE	Epidemiological study	Positive relationship between malaria endemicity a HbE frequency	(Flatz, Pik et al. 1965)
HbC	Case-control studies	80% protection against severe malaria	(Agarwal, Guindo et al. 2000)
		29% reduction in incidence of clinical malaria in heterozygotes	(Modiano, Luoni et al. 2001)

**Table 1.2 Haemoglobinopathies and malaria**  
Summary outlining the known interactions of haemoglobinopathies with malaria (Adapted from (Roberts and Williams 2003))

Although the relationship between the malaria parasite and the erythrocyte has led to most study on the interaction of genetic polymorphisms and susceptibility to or protection from malaria disease, many other genes in different systems and affecting alternative stages of the lifecycle are equally important. As an example of these associations, a large case-control study of malaria in West African children identified a human leucocyte class I antigen (HLA-Bw53) and an HLA class II haplotype (DRB1\*1302-DQB1\*0501), common in West Africans but rare elsewhere, which were independently associated with protection from severe malaria (Hill, Allsopp et al. 1991). Of considerable interest also, are polymorphisms affecting host molecules involved in the pathogenesis of malaria disease. Associations have been described with mutations in CD36, (Aitman, Cooper et al. 2000; Pain, Urban et al. 2001), albeit in opposite directions, intercellular adhesion molecule-1 (ICAM-1), with a specific mutation termed ICAM-1<sup>kilifi</sup> common in Kenya, yet predisposing to severe malaria (Fernandez-Reyes, Craig et al. 1997) in some populations but not others (Grau, Taylor et al. 1989; Kwiatkowski 1990; Bellamy, Ruwende et al. 1999; Kun, Klabunde et al. 1999. Polymorphisms in the promoter region of TNF, increased in severe malaria, (Grau, et al. 1989 ) and implicated in the pathogenesis of murine cerebral malaria, (Grau, Fajardo et al. 1987), have been related to both susceptibility and protection (Knight, Udalova et al. 1999).

### **Innate immunity to malaria**

Reanalysis of experimental *P. falciparum* infections induced in malaria-naïve individuals between 1940 and 1963 as a treatment for neurosyphilis, revealed that the parasitaemia at which parasite growth is controlled is highly predictable within

an individual between first and second infections and between infection with different species (Molineaux, Trauble et al. 2002). It was concluded that this is best explained by innate immune mechanisms employed by the host to limit maximum parasite density. It has been suggested that innate immune mechanisms are triggered by a pre-determined host-specific threshold of parasite density resulting in stimulation of immune effector cells in response to a parasite density in excess of this threshold (Kwiatkowski and Nowak 1991). Possible effector mechanisms include the production of pro-inflammatory cytokines interferon- $\gamma$  (IFN- $\gamma$ ), TNF and various interleukin (IL) molecules including IL-8 and IL-12p70 by peripheral blood mononuclear cells (PBMC). Recent *in vitro* studies support this showing production of TNF, IL-12p70 and IFN- $\gamma$  by PBMC from naïve donors within 12 hours of stimulation by *P. falciparum* infected erythrocytes (Scragg, Hensmann et al. 1999). Furthermore malaria-naïve individuals experimentally infected with *P. falciparum* already had a detectable increase in the levels of INF- $\gamma$ , TNF and IL-8 at the first microscopically detectable appearance of parasites (Hermsen, Konijnenberg et al. 2003).

A recent longitudinal study conducted in Papua New Guinea investigating parasite density regulation in semi-immune children demonstrated control of parasitaemia around a threshold. This threshold was maintained within individuals independent of species or genotype of infecting *Plasmodium* parasite (Bruce and Day 2003). These data and that obtained from the syphilis treatment records suggest the presence of host-specific density-dependent mechanisms irrespective of genotype or species of infecting parasite. The effector cells involved in such interactions and the determinants of their efficacy remain to be fully characterised.

## **Acquired immunity to malaria**

Important observations regarding the nature of acquired immunity to malaria can be gleaned from the epidemiological picture already outlined. In young infants, the resistance displayed towards clinical malaria can be attributed to the transfer of immunity, in the form of humoral factors, from mother to child. This important role for humoral mechanisms of protection is supported by the classical studies describing the passive transfer of antibodies from immune individuals to patients with acute clinical malaria and the resultant resolution of infection (Cohen, McGregor et al. 1961). Following this period of relative protection, children become susceptible to severe disease and death although it is likely that a degree of immunity to severe life-threatening episodes is apparent after only a few disease episodes (Gupta, Snow et al. 1999). In contrast the prevalence of asymptomatic parasitaemia continues to rise beyond the age at which immunity to severe malaria has developed. This and the observation that a seemingly well, asymptotically parasitised child can be susceptible to severe disease have led to the hypothesis that acquired immunity must be directed, at least in part, against polymorphic targets with individuals gaining a repertoire of specificities over time.

The identification and characterisation of the host immune responses and the parasite antigenic targets underlying these epidemiological observations is essential in understanding the interaction of the host and the parasite, an understanding which would in turn better inform the development of an effective malaria vaccine. The studies described in this thesis concentrate on the intra-erythrocytic asexual stage of parasite development and specifically on humoral responses directed against the surface of parasite-infected erythrocytes. This area will form the bulk of the

literature review with a brief summary of immunity to the merozoite stage also discussed.

## **Immunity to the erythrocytic stage**

### **Effector mechanisms against merozoites**

Invasion of erythrocytes by merozoites is an essential component of a patent *P. falciparum* infection. It involves an intricate progression of events linking both parasite proteins and receptors on the erythrocyte. Mediating the initial attachment of the merozoite to the erythrocyte surface are the antigens merozoite surface proteins 1-9 (MSP1-9) (Holder 1994). Successful invasion requires processing of the whole MSP1 molecule until only a 19 kilodalton carboxyl terminus fragment (MSP1<sub>19</sub>) remains on the merozoite (Blackman and Holder 1992; Holder, Blackman et al. 1992). Following attachment the merozoite re-orientates itself until the apical membrane is in contact with the erythrocyte membrane. This process is thought to be facilitated by apical membrane antigen 1 (AMA1), a protein also requiring proteolytic processing (Triglia, Healer et al. 2000; Mitchell, Thomas et al. 2004). Formation of a tight junction between the merozoite and the erythrocyte is then facilitated by the release of myriad antigens including the erythrocyte binding antigens (EBA175, EBA140, EBA180 and EBL-1), (Adams, Blair et al. 2001), and the reticulocyte binding homologues (*Pf*RH1, 2a, 2b, 3 and 4) (Rayner, Galinski et al. 2000; Taylor, Grainger et al. 2002), from organelles at the apical end of the merozoite. Due to the short time these antigens are potentially exposed to the host immune system, it is likely that antibodies are the predominant effector of anti-merozoite immunity. Antibodies could prevent invasion of the erythrocyte thus

preventing the establishment of a symptomatic infection in many ways. These include opsonisation and resultant phagocytosis, complement-mediated damage of merozoites or blockage of erythrocyte-binding sites.

There have been contrasting results from studies investigating whether the presence of antibodies against the antigens listed are associated with protection from clinical malaria. Many studies have suggested that levels of anti-MSP1<sub>19</sub>, anti-MSP1 block 2, anti-MSP2, anti-MSP-3, anti-AMA1 and anti-EBA175 may be associated with protection (Riley, Allen et al. 1992; al-Yaman, Genton et al. 1996; Egan, Morris et al. 1996; Taylor, Allen et al. 1998; Conway, Cavanagh et al. 2000; Okenu, Riley et al. 2000; Meraldi, Nebie et al. 2004; Polley, Mwangi et al. 2004), with yet others showing no association, (Dodoo, Theander et al. 1999; Corran, O'Donnell et al. 2004; Okech, Corran et al. 2004). It is unclear exactly why the disparity in results should exist. It may be they reflect genuine differences in ability of anti-merozoite antigens to protect in different settings or it may simply be a methodological issue highlighting the difficulties with assessing correlates of immunity.

### **Cellular immunity to the erythrocytic stage**

The infected erythrocyte does not express major histocompatibility complex (MHC) molecules and so is incapable of direct antigen presentation, as a result this stage in the life-cycle has generally been thought not to be a target for cell-mediated immunity. Recent data however challenge this. Naïve individuals never previously exposed to *P. falciparum* infection were challenged with a low dose of blood stage parasites and then cured prior to the development of symptoms. It was shown that

although these individuals were immune to subsequent challenge by the same strain of parasite, they developed no detectable antibody response (Pombo, Lawrence et al. 2002). Rather, protected individuals had marked proliferative T-cell responses involving both CD4+ and CD8+ cells, a pro-inflammatory cytokine response involving INF $\gamma$  and high concentrations of inducible nitric oxide in PBMC's. Given that naturally acquired immunity is associated with antibodies to erythrocyte surface variant antigens, (Marsh, Otoo et al. 1989), as will be discussed in some detail, and that cell-mediated immunity is not thought to contribute a great deal to immunity it is feasible that the parasite antigens involved in inducing these apparently protective responses will be under little immune pressure and therefore may not vary. It is interesting to speculate whether or not these mechanisms play a role in protecting young children in endemic areas and whether or not their manipulation could provide additive efficacy for a malaria vaccine targeting the erythrocytic stage. At present the parasite antigens responsible for this response are unknown.

### **Antibody responses to the infected erythrocyte surface**

In 1917 Wagner von Jauregg inoculated tertiary syphilis patients with blood from individuals infected with *Plasmodium vivax* in an attempt to control their disease by raising their body temperature. A chance finding had demonstrated the susceptibility of *Treponema pallidum* to heat and malaria-induced fevers were employed as a means of eliciting this effect *in vivo*. Although much work was done using the non-*falciparum* strains of malaria, important information was acquired regarding the development of immunity to *P. falciparum* in humans, reviewed in (Desowitz 1991). By infecting patients with *P. falciparum* isolates from distinct geographical areas and by re-infecting using alternative species of malaria it became apparent that to a

degree, development of immunity was both species and, within *P. falciparum*, strain specific (Jeffery 1966; Collins and Jeffery 1999; Collins and Jeffery 1999).

It was the development of schizont-infected cell agglutination (SICA) in 1938 that allowed investigation of antibody responses directed at the infected erythrocyte surface during the asexual erythrocyte stage of the parasite life cycle (Eaton 1938). As discussed, malarial parasites have apparently evolved a perfect ecological niche. Their propensity to reside within the erythrocyte during the asexual growth phase of the parasite life cycle allowed them ostensible shelter from the host's immune response. As mentioned, erythrocytes lack any surface MHC molecules and thus have no mechanism for antigen presentation. However, with the development of the SICA assay, Eaton showed that the actual situation was rather more complicated. When the asexual intraerythrocytic parasites of *P. knowlesi* had matured to the schizont stage, the infected erythrocytes could be agglutinated by sera from rhesus monkeys previously infected with *P. knowlesi* but not by sera from non-immune monkeys. In contrast, uninfected rhesus monkey erythrocytes or erythrocytes containing immature asexual parasites were not agglutinated by immune sera (Eaton 1938). That this reaction only occurred with the more mature intraerythrocytic parasite stages implied the presence of stage-specific parasite-induced antigens on the surface of the red cell.

Over the ensuing decades the lack of ability to culture *P. falciparum* parasites *in vitro* necessitated the use of animal models in the attempt to elucidate the complex steps in the acquisition of anti-malarial immunity. In 1965, Brown and colleagues demonstrated elegantly the variant-specificity of the antibody response directed



against the infected erythrocyte (Brown and Brown 1965). Utilising the SICA test with *P. knowlesi* parasites in rhesus monkeys, they showed that recrudescence parasites from a single inoculum differed in the antigens they expressed on the surface of the infected erythrocyte. Each wave of parasitaemia was subsequently followed by the presence of antibodies specific to the wave. In a series of influential papers this group demonstrated that chronic infections in *P. knowlesi* malaria were apparently maintained by the serial expression of different antigenic types. They subsequently showed that whilst protective immunity could not be inferred from the presence of SICA antibodies alone, suggesting that these antibodies were just a marker of exposure or a downstream marker of a protective response, protection was found to be correlated with the presence of variant-specific opsonising antibodies directed against the infected erythrocyte (Brown 1971; Brown 1973; Brown and Hills 1974).

As these initial experiments were not done with cloned parasite populations, it was conceivable that the appearance of different variants during the course of a chronic infection could be due to immune selection by sera of sub-populations of parasites present within the infecting parasite culture. In 1983, Barnwell and colleagues conclusively showed this was not the case when they performed similar experiments using a cloned line of *P. knowlesi* (Barnwell, Howard et al. 1983).

Together, data from experimental models and that inferred from the treatment of tertiary syphilis patients, pointed strongly to the development of anti-malarial immunity being predominantly variant-specific. However, it was also evident from these studies that the density of parasitaemia reduced and the period between

recrudescences became more prolonged with the appearance of each new variant throughout the course of a chronic infection. This was not a result of a reduction in parasite virulence as the infection progressed, as, when a new variant was transferred into a naïve host, they demonstrated the same degree of virulence in terms of maximum parasitaemia, pre-patent period and clinical symptoms as the original inoculating parasite had in the original host. This observation suggests some degree of variant-transcending immunity may exist. This may be due to the induction of cross-reactive antibodies to as yet undetermined targets, the development of humoral responses against conserved non-variant antigens or even improved variant-specific responses perhaps through increased T-cell help.

#### **Human antibody responses to *P. falciparum*-infected erythrocytes**

Studies on the human antibody response to the infected erythrocyte surface were made possible by two scientific advances: the first being the successful culturing of *P. falciparum* parasites *in vitro*, (Trager and Jensen 1976), and the second, the development of assays directly measuring antibodies from human sera reacting against the infected erythrocyte. These assays included antibody-mediated microagglutination similar to the SICA test already described, indirect surface-labelled immunofluorescence and adhesion-inhibition on endothelial cells (Sherwood, Marsh et al. 1985) (Marsh, Sherwood et al. 1986) (Udeinya, Miller et al. 1983). As with the SICA test, it was demonstrated that microagglutination of *P. falciparum*-infected erythrocytes only occurred with mature infected erythrocytes, specifically late trophozoite and schizont stages. Agglutination occurred in a predominantly strain-specific manner and was only mediated by sera from malaria-exposed donors (Marsh, Sherwood et al. 1986; Forsyth, Philip et al. 1989; Marsh,

Otoo et al. 1989; Southwell, Brown et al. 1989; Newbold, Pinches et al. 1992; Smith, Chitnis et al. 1995). Adhesion inhibition to endothelial cells also occurs in a strain-specific manner and as with agglutination only occurred with sera from malaria-exposed donors (Udeinya, Miller et al. 1983). In a direct comparison there was good correlation demonstrated between indirect surface-labelled immunofluorescence and agglutination (Marsh, Sherwood et al. 1986). Although the agglutination reaction is still widely used as a means of assessing antibody reactivity towards the infected erythrocyte, more recently flow cytometry has been employed as a means of measuring the anti-infected erythrocyte surface antibody response (Piper, Roberts et al. 1999; Staalsoe, Giha et al. 1999; Williams and Newbold 2003). As with agglutination, sera from malaria-exposed but not naïve donors label the infected erythrocyte surface. In addition, sera from donors resident within endemic areas only label mature infected erythrocytes but not uninfected erythrocytes or erythrocytes infected with immature parasites. When individual parasite isolates were used to deplete sera of isolate-specific antibodies, reactivity against the isolate used for depletion was reduced whereas reactivity against other isolates remained unaltered, demonstrating the strain-specificity of the reactivity measured (Staalsoe, Giha et al. 1999). It is important to note however that both for agglutination and flow cytometry the precise antigenic target on the surface of the infected red blood cell is unknown. Although likely, it is not absolutely certain that both assays are measuring responses to the same antigen.

The ability of humans to mount an antibody response to the infected erythrocyte was demonstrated in 1986 (Marsh and Howard 1986). Children resident in The Gambia naturally infected with *P. falciparum*, developed isolate-specific agglutinating

antibody responses against the surface of their own infected erythrocytes during convalescence, antibodies absent at the time of the acute infection. Sera from uninfected adults from the same area agglutinated a panel of parasite isolates in a non-specific manner. These results raised the possibility that if anti-infected erythrocyte antibody responses are important in the development of anti-malarial immunity, then perhaps adults develop variant-specific immunity towards the erythrocyte surface through being exposed to multiple isolates over the course of their childhood and adolescence, so-called 'piecemeal' acquisition of antibody responses. Equally it is possible that some degree of cross-reactivity exists between anti-surface responses, allowing adults recognition of conserved, less immunogenic epitopes present on the infected-erythrocyte surface.

In an attempt to answer this question, serum from a Gambian adult was used to elute antibodies from the surface of infected erythrocytes, obtained from a symptomatically infected child. These isolate-specific antibodies agglutinated a panel of isolates from children. Serum adsorbed against the same isolate, did not agglutinate any of the panel (Marsh and Howard 1986). Although these results suggested an element of cross-reactivity within the adult's antibody response or recognition of more conserved epitopes, it must be recognised that while one variant may predominate within a natural infection, the likelihood is that there will be many variants present in one single inoculum (Marsh and Howard 1986).

Newbold and colleagues questioned the possibility of the presence of cross-reactive or conserved epitopes on the surface of the infected erythrocyte in 1992, (Newbold, Pinches et al. 1992). Using a differential staining technique, they demonstrated that a

mixture of field and laboratory-cultured isolates very rarely formed mixed agglutinates when incubated with immune serum. This suggested that the antibody response was predominantly variant-specific.

As illustrated in the earlier studies involving *P.knowlesi*-infected rhesus monkeys, (Brown and Brown 1965; Brown, Brown et al. 1968), and as can be inferred from the epidemiology of malaria infection and disease in humans, an element of both variant-specific and transcending responses must co-exist. In those resident in malaria-endemic areas, the observation of the apparently healthy, asymptotically parasitised child who subsequently dies from severe malaria can be satisfactorily explained by the presence of variant-specific immunity playing a role in protection, with the child becoming infected with a 'new variant' or heterologous genotype previously not encountered (Collins and Jeffery 1999). Conversely, variant-transcending immunity, whether against the infected-erythrocyte surface or to another non-variant antigen, is suggested by the severity of clinical disease reducing with time and exposure.

In addition to the studies by Marsh and Newbold, variant-specific 'piecemeal' acquisition of antibody responses has been illustrated in several different epidemiological settings (Jeffery 1966; Hommel, David et al. 1982; Bull, Lowe et al. 1998; Giha, Theander et al. 1998; Giha, Staalsoe et al. 1999; Giha, Staalsoe et al. 2000). As mentioned, deliberate human infections resulted in immunity to both homologous and heterologous *P. falciparum* secondary infections, however, a current patent infection did not prevent the development of a clinically symptomatic response following infection with a heterologous strain of *P. falciparum* (Collins and

Jeffery 1999). In fact, individuals infected with heterologous secondary infections displayed less clinical immunity, as measured by duration and extent of fever, than they did in response to recrudescent parasitaemia during a chronic infection (Collins and Jeffery 1999). In 1982, Hommel and colleagues demonstrated that convalescent sera from squirrel monkeys experimentally infected with *P. falciparum* recognised the infecting homologous parasite only (Hommel, David et al. 1982). Furthermore antibodies that blocked the cytoadherence of *P. falciparum*-erythrocytes to amelanotic melanoma cells were shown to be isolate-specific (Udeinya, Miller et al. 1983). In Papua New Guinea, children developed variant-specific antibodies specific to the infecting parasite isolate during convalescence, with both the proportion of isolates recognised and the intensity of recognition increasing with age (Forsyth, Philip et al. 1989). Adults in the same study agglutinated a significantly wider range of isolates than did children. In Pakistan, only three out of fifteen acute sera from infected children agglutinated their infecting isolate. In contrast all fifteen convalescent sera agglutinated the corresponding infecting isolate. A panel of 10 hyperimmune adults from the same region not only recognised the majority of the isolates tested but recognised them to a greater degree (Iqbal, Perlmann et al. 1993). However, in the studies from Papua New Guinea and from Pakistan, none of the adult sera recognised all of the isolates tested, suggesting that rather than recognition of conserved epitopes on the infected erythrocyte surface adults developed immunity through successive exposures to multiple parasite variants, and, that although it is possible that cross-reactivity exists, responses are not pan-specific.

More recently in Kenya, a longitudinal study demonstrated that homologous serum taken before the disease episode, rarely agglutinated parasite isolates taken from

malaria cases at the time of acute disease. In contrast isolates not involved in the disease-causing episode were readily recognised (Bull, Lowe et al. 1998). This implied that only parasite isolates to which the host does not already possess a specific antibody response could cause disease, supporting the notion of a gradual acquisition of variant-specific immunity.

In an area of low endemicity in Sudan, agglutinating antibody responses displayed marked seasonal variation (Giha, Theander et al. 1998) (Giha, Staalsoe et al. 1999). An individual's ability to agglutinate a panel of isolates at the end of a high transmission season was not affected by that individual's preceding malaria history. Those individuals apparently not suffering clinical malaria during the season exhibited the same increase in recognition following exposure during the high transmission period (Giha, Theander et al. 1998). Furthermore, individuals followed over many years for their ability to recognise the infected erythrocyte surface, displayed marked variation over time. Clinical disease resulted in a marked and specific antibody response that decayed over some months with overall neither the disease history of any individual nor the number of episodes of asymptomatic parasitaemia showing any relationship with the number of isolates recognised (Giha, Staalsoe et al. 1999). This suggests that either the target antigens are extremely polymorphic from one high transmission season to the next, or the seasonal component of the agglutinating antibody response is of short duration and targets relatively conserved epitopes. A recent longitudinal study in Kenya describing the kinetics of antibody responses to infecting isolates amongst young children demonstrated considerable variation in the ability of an individual to make an adequate humoral response and maintain it (Kinyanjui, Bull et al. 2003). Whether

this variability in response is due to specific innate host factors or is a consequence of the particular infecting isolate is unknown.

Thus perhaps this well-illustrated diversity of serological responses to the infected red blood cell is not limitless. The adsorption and elution experiments described from the Gambia, (Marsh and Howard 1986), identified antibodies from a selection of adults which recognised all isolates tested, suggesting a conserved red cell surface expressed antigen. Moreover immune sera from adults from East and West Africa, South America and South-east Asia agglutinated to varying degrees, erythrocytes infected with parasites from East and West Africa (Aguilar, Albrecht et al. 1992). This result is somewhat similar to the classical passive transfer experiments whereby immunoglobulin from immune African adults dramatically reduced parasitaemia in distant regions (Cohen, McGregor et al. 1961) (Edozien, Gilles et al. 1962; Sabchareon, Burnouf et al. 1991). A more recent study likewise documented that antibody reactivity towards the infected red blood cell surface depended predominantly on the transmission characteristics of the site of plasma collection but was completely independent of the geographical origin of the parasites (Nielsen, Vestergaard et al. 2004). The serological variability of pRBC surface expressed antigens may thus be finite.

The induction of antibodies during an acute episode to parasite isolates apparently not involved in that episode has been suggested as evidence for cross-reactivity of responses against the pRBC surface (Giha, Theander et al. 1998; Chattopadhyay, Sharma et al. 2003). Chattopadhyay and colleagues in India, demonstrated using the mixed agglutination assay that 20-30% of agglutinates between different wild



isolates were of mixed colour and from this concluded that there must be common epitopes or cross-reactivity of responses (Chattopadhyay, Sharma et al. 2003). Although this supports earlier findings by Marsh and Howard, (Marsh and Howard 1986), caution in interpretation must be advised. As Roberts alluded to in a related editorial, the chance of heterologous recognition of different variant types is directly related to the proportion of minor variant types within each parasite population expressed through antigenic switching. Thus even a low degree of cross-reactivity between isolates could result in a high proportion of mixed agglutinates (Smith, Chitnis et al. 1995; Roberts 2003). In addition, although one variant may dominate in an acute infection, several other variants may well be present; this could lead to induction of antibodies to apparently heterologous isolates. Furthermore it may be that in both India and in Sudan, where these studies were done, the extent of variant surface antigen variability is more limited than that seen in other areas in sub-Saharan Africa where transmission is higher. Certainly this cross-induction of responses was not seen to the same extent in other areas with higher transmission characteristics (Marsh and Howard 1986; Iqbal, Perlmann et al. 1993; Reeder, Rogerson et al. 1994; Bull, Lowe et al. 1999).

Several studies have suggested that there may be a subset of serologically distinct variant isolates (Bull, Lowe et al. 1999; Bull, Kortok et al. 2000; Nielsen, Staalsoe et al. 2002). Initial studies in Kenya indicated that parasites varied considerably in their frequency of recognition by antibodies from children. A proportion of isolates displayed high frequency of recognition by children and this was independently associated with more severe disease and younger host age (Bull, Lowe et al. 1999; Bull, Kortok et al. 2000). This association has been confirmed in other areas

(Nielsen, Staalsoe et al. 2002; Lindenthal, Kremsner et al. 2003). Those parasites less frequently recognised tended to come from older children with uncomplicated, milder disease. It would appear that the antigenic determinants of recognition amongst the frequently recognised parasites represent a less diverse group of variant surface antigens, with the size of the agglutinate formed being positively correlated with the degree of recognition (Bull, Kortok et al. 2000). The degree of recognition of an isolate on the other hand is not related to the number of parasite genotypes present in the infection (Bull, Kortok et al. 2000). More recently it has been shown that the more frequent recognition of parasites causing severe disease cannot be explained in terms of the antibody repertoire at the time of disease. In a recent analysis of data from Kenya, there was no significant difference between those children with non-severe disease and those with severe disease in terms of their anti-erythrocyte surface antibody repertoire at the time of acute disease (Bull P.C. *in press*), a finding also seen amongst children in The Gambia (Erunkulu, Hill et al. 1992). Thus if children who suffer an episode of severe disease are in some way less immune than those who don't it is not reflected in their heterologous anti-variant surface antigen antibody repertoire at the time of acute disease.

### **Anti-infected erythrocyte antibodies and protection from malaria**

Studies investigating which immune responses to the infected erythrocyte confer protection against clinical malaria are problematic. Children residing in malaria-endemic areas are constantly challenged by *P. falciparum* and are thus frequently infected. Because of the ubiquity of infection children develop antibody responses to a wide range of parasite antigens, the range of which increases with age. Differentiating those responses that are protective from those simply reflecting

exposure has proven difficult. The first study to definitively show an association between antibodies to the infected erythrocyte and protection from clinical disease was carried out by Marsh and colleagues in 1989 (Marsh, Otoo et al. 1989). Agglutinating antibodies directed against the surface of a randomly selected wild isolate was the only immunological assay associated with protection from subsequent clinical malaria. Since then studies examining humoral responses against the erythrocyte-surface and their association with protection from clinical disease have yielded conflicting results. It is clear that following infection individuals develop antibodies specific to the infecting isolate (Bull, Lowe et al. 1998; Giha, Staalsoe et al. 2000; Chattopadhyay, Sharma et al. 2003). What is less clear is how relatively cross-reactive responses, assessed using heterologous isolates, are involved in protection from disease. In Kenya, the ability of sera to agglutinate a randomly selected isolate was not associated with protection (Bull, Lowe et al. 1998; Bull, Lowe et al. 2002). However in Sudan, antibodies directed against an isolate from Ghana were associated with protection while antibodies to six Sudanese isolates were not. Similarly, in Ghana responses to a Sudanese isolate and to a Ghanaian isolate demonstrated an association with protection but responses to another Ghanaian isolate did not (Giha, Staalsoe et al. 2000; Dodoo, Staalsoe et al. 2001). This data together with the results from the Gambia already mentioned (Marsh, Otoo et al. 1989); suggest that responses to some isolates but not others are associated with protection. It may be that antibodies directed against these isolates are genuinely more protective or that these isolates are in some way different in the antigens expressed. They may express to a greater degree a cross-reactive or conserved sub-set of variant antigens. Alternatively, these results may simply reflect

differences in prevalence and the ability of these measured responses to act as surrogate markers of protection.

It should be noted however that in none of these studies was asymptomatic parasitaemia taken into account at the time the sera were assayed. Several studies have demonstrated an association between *P. falciparum* infection and enhanced anti-pRBC antibody responses to heterologous isolates (Iqbal, Perlmann et al. 1993; Giha, Staalsoe et al. 1999; Giha, Staalsoe et al. 1999; Ofori, Doodoo et al. 2002) In support of this, more recent data from Kenya demonstrated that the proportion of heterologous isolates agglutinated was strikingly higher amongst children with a microscopically detectable parasitaemia at the time of assay compared to those without and that this association was not just due to cumulative exposure (Bull, Lowe et al. 2002). As the sera in this study were assayed at the end of a dry season these asymptomatic parasite infections will have most likely been sustained as chronic infections since the preceding rainy season. It can be conjectured that a degree of cross reactivity may exist within the antibody repertoire induced by such infections, with the constant presence of a range of antigenic variants over time serving to continuously boost the otherwise rapidly decaying antibody response. Alternatively, it may be that these chronic infections have had longer to switch antigenic variants displayed thus inducing a broader range of antibody specificities.

A subsequent study using flow cytometry again demonstrated an enlarged repertoire of anti-infected red blood cell surface antibodies amongst those children with a microscopically detectable parasitaemia (Kinyanjui, Mwangi et al. 2004). Furthermore the presence of asymptomatic parasitaemia and anti-infected

erythrocyte antibodies directed at heterologous isolates together was associated with protection against subsequent clinical malaria compared to either alone (Kinyanjui, Mwangi et al. 2004), a finding also seen with severe malaria (Bull, Lowe et al. 2002). Independently both the presence of heterologous anti-surface antibodies in the absence of parasites and the presence of parasites with no concurrent heterologous antibody response were associated with susceptibility to clinical malaria during the subsequent six months. The mechanisms underlying these associations are presently unknown. It may be that the development of immunity against clinical malaria is associated with the ability to maintain chronic asymptomatic infections. This hypothesis is supported by the observation that, with age, disease incidence decreases at a time when the prevalence of parasitaemia increases (Marsh 1992). The increased susceptibility observed in those with parasites and poor concomitant heterologous antibody responses might reflect a poor inherent ability to respond appropriately. Such children have been reported in a number of studies (Giha, Staalsoe et al. 1999; Ofori, Dodoo et al. 2002; Kinyanjui, Bull et al. 2003) although on individual basis it is not known if these children are actually more susceptible to clinical malaria. Having antibodies in the absence of detectable parasitaemia may simply indicate a recently treated symptomatic infection and may thus be reflective of a sub-population of individuals at increased risk in terms of either challenge or inherent host susceptibility factors. Of course these differences in susceptibility may simply be highlighting differences in exposure and thus risk between the groups. Those with parasites at the time of sampling have a defined exposure, whereas those without parasites will be a more heterogeneous group in terms of likelihood of risk and thus any difference in protection or susceptibility may be more difficult to disentangle.

It seems clear that following repeated challenge individuals become progressively better equipped to handle a larger proportion of the possible variants they are exposed to. This process is undoubtedly complex and the resultant functional immunity will consist of many components. It would appear though, from epidemiological and modelling data that the development of anti-disease immunity occurs faster than would be expected if it purely depended on encountering all possible locally relevant variants (Gupta, Snow et al. 1999). It is likely that responses to different stages of the life-cycle will be additionally and simultaneously relevant and that as well as a strong and protective variant-specific response there will be both an element of cross-protection amongst selected antigens and possibly a slower to develop but functionally extremely important response against less immunogenic conserved targets.

### **Antigenic targets on the *P. falciparum* -infected red blood cell surface**

The isolate-specific agglutination of infected erythrocytes by sera from malaria-immune hosts along with the observation that immune sera did not agglutinate uninfected or immature-infected erythrocytes, led to the hypothesis that there must be new, malaria-specific antigens expressed on the surface of infected erythrocytes (Eaton 1938). This finding as well as the observation that only young ring-stage infected-erythrocytes were present in the peripheral blood of individuals infected with *P. falciparum* led to the understanding that the parasite must modify the surface of the erythrocyte it invades. Since this became apparent, the challenge to identify, sequence and understand the function of these surface-exposed molecules has proven not only demanding but also important. Many parasite-induced proteins and altered

host proteins have been identified on the surface of *P. falciparum* infected erythrocytes including RIFIN, STEVOR, SURFIN and altered band 3 (Cheng, Cloonan et al. 1998; Kyes, Rowe et al. 1999) (Crandall, Collins et al. 1993; Winter, Kawai et al. 2005). However, the best characterised and most studied of these proteins is *Plasmodium falciparum* erythrocyte membrane protein-1 (*PfEMP1*).

### ***PfEMP1***

In 1984, Leech and colleagues identified a high molecular weight, strain-specific protein present on the surface of *P. falciparum*-infected erythrocytes (Leech, Barnwell et al. 1984). Using Camp and St Lucia strains of parasites, a 200-250kDa weight protein was specifically immuno-precipitated by immune sera from Aotus monkeys infected with the homologous strain of parasites. Sera from monkeys infected with a heterologous strain did not immuno-precipitate the same protein. These surface-exposed proteins were sensitive to low concentrations of trypsin and the antibodies which resulted in their immuno-precipitation blocked binding of infected erythrocytes to melanoma cells in a strain-specific manner (Leech, Barnwell et al. 1984; Howard, Barnwell et al. 1988). The protein was not extracted from infected erythrocytes by a non-ionic detergent such as Triton X-100 but was extracted with sodium dodecyl sulphate (SDS), suggesting linkage to the erythrocyte cytoskeleton (Aley, Sherwood et al. 1984). This family of proteins became known as *Plasmodium falciparum* erythrocyte membrane protein 1 (*PfEMP1*).

*PfEMP1* has been implicated in two key phenomena responsible for much of the pathology associated with *P. falciparum* infections: cytoadherence of pRBC, including rosetting of uninfected erythrocytes and immunoregulatory effects on host

immune effector cells through binding to other host effector molecules and antigenic variation with resultant immune evasion

Erythrocytes infected with the mature forms of *P. falciparum* disappear from the peripheral circulation, a phenomenon known as sequestration (Bignami and Bastianelli 1889). Sequestration results from the cytoadherence of infected erythrocytes to a variety of host receptors situated on endothelial cells in most organs of the human body (Miller 1969; Luse and Miller 1971; Gilks, Walliker et al. 1990). *PfEMP1* is associated temporally with the appearance of knob-like protrusions on the pRBC surface, (Leech, Barnwell et al. 1984), knobs themselves being associated with the expression of *PfEMP1* (Aley, Sherwood et al. 1984; Leech, Barnwell et al. 1984). Erythrocytes bearing more mature parasites attach themselves to endothelial cells via these knobs (Luse and Miller 1971; Kilejian 1979; MacPherson, Warrell et al. 1985). However it must be noted that knobless laboratory cloned isolates have also been shown to bind to endothelial cell receptors and a protein with the characteristics of *PfEMP1* has been immunoprecipitated from the surface of these knobless clones (Biggs, Gooze et al. 1990). In fact recent data has highlighted that in the absence of knobs *PfEMP1* clusters in a similar way although around 50% less is expressed on the surface of infected erythrocytes (Horrocks, Pinches et al. 2005). This finding supports earlier work showing that although adherence to CD36 (a characteristic attributable to *PfEMP1*) is maintained in the absence of knobs, the attachment is much weaker and more easily disrupted under flow (Crabb, Cooke et al. 1997).

There is now overwhelming evidence that *PfEMP1* is the principal parasite receptor mediating cytoadherence. In 1996 Gardner and colleagues described the abrogation



of binding of various parasite clones to a number of previously described endothelial cell receptors through pre-treatment of mature pRBC with trypsin, thus demonstrating that the parasite receptor mediating cytoadhesion had characteristics similar to those of *PfEMP1* (Gardner, Pinches et al. 1996). Conclusive evidence for the role of *PfEMP1* in sequestration came when affinity purification of detergent extracts of radio-iodinated infected cells using known host endothelial cell receptors, ICAM-1, CD36 and TSP, yielded proteins with all the properties of *PfEMP1* (Baruch, Gormely et al. 1996).

Simian malaria models were the first to provide evidence for antigenic variation (Brown and Brown 1965; Brown, Brown et al. 1968; Brown 1973; Barnwell, Howard et al. 1983; Handunnetti, Mendis et al. 1987). The first direct evidence that *P. falciparum* also underwent antigenic variation came in 1983 by Hommel and colleagues (Hommel, David et al. 1983). By infecting Saimiri monkeys with *P. falciparum* and serotyping the resultant peaks of parasitaemia using indirect surface immunofluorescence, they demonstrated that each wave of parasitaemia was associated with antigenically distinct determinants present on the surface of the *P. falciparum*-infected red blood cells. Studies with cloned parasites demonstrated that antigenic variants arose with extraordinary frequency, switching away from an existing antigenic and adhesive phenotype at a rate of up to 2% per generation in the absence of immune selection (Roberts, Craig et al. 1992).

A subsequent series of experiments linked the antigenic and adhesive properties of *P. falciparum*-infected erythrocytes with the modulation of *PfEMP1*. In a family of sub-clones derived from a parental clone that displayed binding to ICAM-1, changes in the capacity to bind to ICAM-1 within the daughter clones were always associated

with a change in antigenic type (Roberts, Craig et al. 1992). In a separate set of experiments, parasites derived from a single clone were selected for their capacity to bind to endothelial cells. These selected parasite lines reacted with agglutinating antiserum that failed to react with the parental clone. Immunoprecipitation with this agglutinating serum demonstrated that the selected daughter clones expressed a larger and antigenically discrete variant of *PfEMP1* that was cross-reactive amongst the daughter clones but distinct from that expressed by the parental clone (Biggs, Anders et al. 1992). A fuller understanding of these pathogenic processes requires an in depth understanding of the genetic control mechanisms underlying transcription and expression of this family of proteins.

### ***Var* genes**

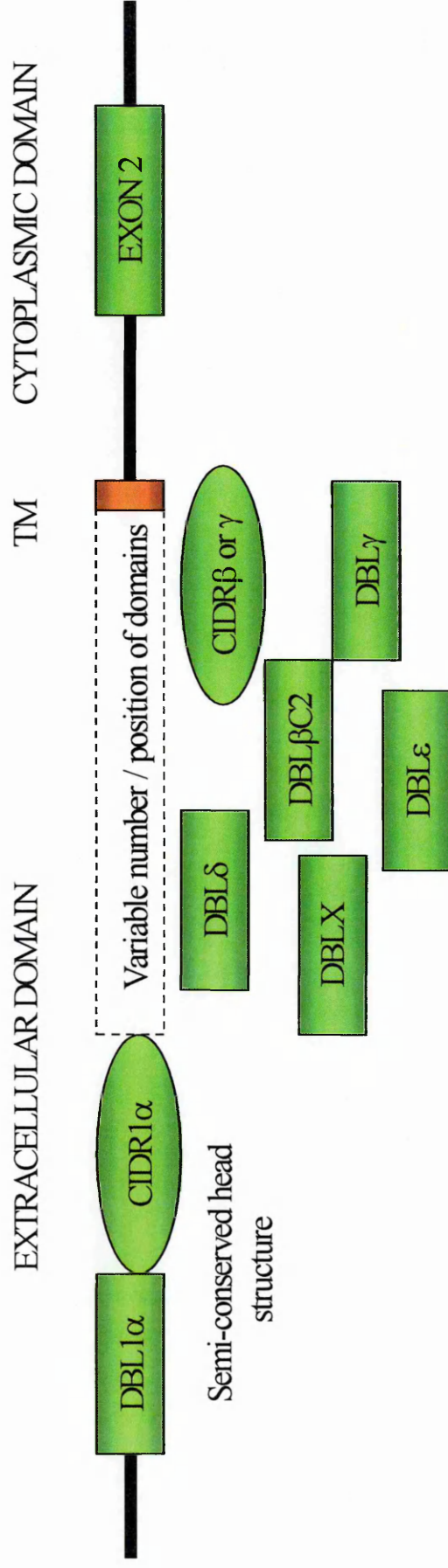
*PfEMP1* is encoded by a family of genes called *var* (Baruch, Pasloske et al. 1995; Smith, Chitnis et al. 1995; Su, Heatwole et al. 1995). The finding that within each haploid *P. falciparum* genome there are around 60 copies of these genes, that dramatic sequence variation exists among the gene copies, and that the encoded protein domains all exhibited receptor binding properties firmly established the *var* genes and their products as the major determinants of cytoadherence and antigenic variation within the *P. falciparum* genome (Baruch, Pasloske et al. 1995; Smith, Chitnis et al. 1995; Su, Heatwole et al. 1995).

The resultant encoded proteins are mostly extracellular. They exhibit a domain structure and the domains bear homology to the cysteine-rich binding domains of varied *Plasmodium* molecules involved in the binding to and invasion of erythrocytes. These include EBA-175, the *P. falciparum* glycophorin A receptor and

the *Plasmodium vivax* and *Plasmodium knowlesi* ligands that allow invasion of Duffy blood-group positive erythrocytes (Adams, Sim et al. 1992; Chitnis and Miller 1994; Sim, Chitnis et al. 1994). It was for this latter reason these *Pf*EMP1 domains were termed Duffy-binding like domains (DBL). They are interspersed with regions containing multiple cysteine residues termed the cysteine-rich interdomain regions (CIDR).

*Var* genes are encoded in two exons, (figure 1.6). The first exon encodes the exposed extracellular region and the transmembrane domain. It is highly variable both in length and sequence and encodes between 2 and 7 DBL domains and, in the majority of cases, at least one CIDR domain. The second exon, in contrast, is relatively short, highly conserved and encodes the intracellular domain or acidic terminal segment. This segment is hypothesized to anchor the protein to the infected erythrocyte cytoskeleton (Oh, Voigt et al. 2000; Waller, Nunomura et al. 2002). In a recent study investigating the relatedness of DBL domains within different *Pf*EMP1, sequences from 20 *Pf*EMP1 variants were analysed and 65 DBL domains identified (Smith, Subramanian et al. 2000). A phylogenetic tree was constructed using the sequences of these DBL domains along with the DBL domains of EBA-175 of *P. falciparum*, *P. knowlesi* and *P. vivax*. Within this analysis the DBL domains of the EBA proteins grouped closely together, with the DBL domains of *P. knowlesi* and *P. vivax* EBA being very closely related. By comparison the *Pf*EMP1 DBL domains have diverged significantly from their probable ancestral origin and within themselves grouped into five statistically diverse sequence groups. These groups were named  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  and  $\epsilon$ . Using a similar methodology, three classes of CIDR were identified,  $\alpha$ ,  $\beta$  and  $\gamma$  (Smith, Subramanian et al. 2000). A small number of

domains present within the genome studied, *P. falciparum* clone 3D7, did not fit well into the existing classification and as a result these were termed DBL-x or CIDR-x (Gardner, Hall et al. 2002). Despite the sequence signatures allowing each domain type to be classified, there was little sequence homology even amongst domains of the same type. In fact, the sequence similarity overall of the *var* genes encoded within the 3D7 genome ranges from 24-98% (Flick and Chen 2004). *Var* genes are present on all 14 chromosomes of the genome of *P. falciparum* clone 3D7. 24 are situated at the telomere and with two exceptions all the sub-telomeric *var* genes are transcribed towards the centromere. The two exceptions, situated on chromosomes 7 and 11 are oriented towards the telomere. Also oriented towards the telomere is a *var* pseudo-gene situated on chromosome 5 (Gardner, Hall et al. 2002). This gene, known as *3D7chr5var2*, consists of a truncated exon 1 with a deletion at the end of DBL7 $\epsilon$  and no intron or exon 2 intracellular sequences making it unlikely that this particular *var* gene will encode a functional protein. Despite this, it caused considerable interest subsequent to the publication of the genome. The 5' end of this *var* pseudogene was shown to be highly expressed amongst CSA-binding isolates and within wild isolates taken from children suffering from malaria (Rowe, Kyes et al. 2002; Winter, Chen et al. 2003). Such an observation might suggest it has an important function although precisely what this is is currently unknown. It would seem that as not all isolates have such a *var* homologue, it must not be essential for survival (Winter, Chen et al. 2003).



**Figure 1.6**

**Composite *PfEMP1* protein**

Shown is the semi-conserved 5' head structure consisting of DBL1 $\alpha$  and CIDR1 $\alpha$ , following this each *PfEMP1* protein is structurally different and is composed of a variable number of subsequent DBL and CIDR domains. TM refers to the trans-membrane region with EXON2 the highly conserved intracellular portion of this protein. (Adapted from Horrocks et al., 2005)

A further 12 *var* genes are located near the telomere with the remainder forming internal clusters on chromosomes 4, 6, 7, 8 and 12. Analysis of sequences 1.5kb upstream of the *var* genes has revealed three distinct and conserved flanking regions of sequences, nominally upsA, B and C (Gardner, Hall et al. 2002; Kraemer and Smith 2003; Lavstsen, Salanti et al. 2003). These regions show preferential association with different *var* genes, upsA associated *var* genes are always sub-telomeric, upsC *var* genes are always chromosome-internal and upsB *var* genes can be situated in either location (Voss, Thompson et al. 2000). Additionally, transcription of upsA *var* genes is always towards the telomere and upsB associated *var* genes away from the telomere. Internal upsB and C associated *vars* are arranged in head to tail clusters and within the 3D7 genome there are single copies of separate upsD and E promoters associated with unusually conserved single-copy *var* genes.

More recently a classification of *var* genes was proposed taking into account chromosomal location, transcriptional direction, domain structure of the encoded proteins and sequence similarity among coding and non-coding regions (Lavstsen, Salanti et al. 2003). They were divided into three discrete groups, A, B and C with a further two groups B/A and B/C, representing those genes whose characteristics fall between the three groups.

Those genes in group A represent *var* genes encoding large and complex *Pf*EMP1 proteins. The *var* genes in groups B and C bind to CD36 via CIDR, whereas recombinant CIDR proteins originating from group A *var* gene sequences do not bind CD36 (Robinson, Welch et al. 2003). The largest *var* group in 3D7 constitute

the group B genes. These comprise 22 genes all but one of which are situated in the telomeric region. They all share a conserved 5' upsB region. The 13 genes of group C are all located internally.

The *var* gene repertoire of around 60 copies per haploid genome is relatively small compared to multigene families of other eukaryotic protozoa involved in antigenic variation (Rudenko 1999). It is therefore not surprising that large sequence diversity exists between different *P. falciparum* isolates (Su, Heatwole et al. 1995). Using *in situ* hybridisation techniques it was demonstrated that genetically diverse parasites contained essentially, although not completely, non-overlapping *var* gene repertoires (Freitas-Junior, Bottius et al. 2000). This proposed discrete strain structure of *var* gene repertoires, whilst indicative of the extreme diversity of *var* genes, is in accordance with modelling work whereby immune pressure results in genomic organisation into stable antigenic types (Gupta and Anderson 1999). The small extent of overlap that exists, blocks of between 20 and 80 amino acids, between sequences of both laboratory-strains and wild isolates suggests that recombinational reshuffling of *var* genes also exists (Ward, Clottey et al. 1999; Taylor, Kyes et al. 2000). As has been described, the majority of *var* genes are located at the telomeric ends of chromosomes, (Gardner, Hall et al. 2002). It has also been demonstrated, using fluorescence *in situ* hybridisation, that within the *P. falciparum* nuclei the chromosome ends cluster at the periphery (Freitas-Junior, Bottius et al. 2000). This alignment of chromosome ends will physically position non-homologous chromosomes in close proximity with one another so providing a structural set-up for recombination events in this area. This proposed telomere-mediated enhancement of

recombination would have the effect of increasing the relatively modest *var* gene repertoire available to the parasite.

### **Regulation of *var* gene expression**

Using clonal parasite lines selected for specific phenotypes and thus specific expressed *Pf*EMP1 proteins, a number of important findings regarding *var* gene expression and control were elucidated. Firstly, expression of *var* genes is temporally regulated during parasite development. Secondly, telomeric as well as internal *var* genes located on different chromosomes can be selected for expression. Thirdly, the expression of *var* genes is mutually exclusive, i.e. only one member of the *var* gene family is expressed on the surface of an individual infected erythrocyte (Chen, Fernandez et al. 1998; Scherf, Hernandez-Rivas et al. 1998; Kyes, Pinches et al. 2000).

The expression of *Pf*EMP1 is developmentally regulated with the protein appearing on the surface of the infected erythrocyte around 16-18 hours post-invasion coincident with cytoadhesion (Gardner, Pinches et al. 1996). It would appear that the control of *var* gene transcription is also tightly regulated and stage-specific. Two different experimental approaches investigating this issue have led to two opposing hypotheses. Using Northern blot analysis it was shown that the major *var* mRNA transcript encoding the *Pf*EMP1 destined for expression on the erythrocyte surface was present at maximum amounts in late-ring stages declining until barely detectable by the trophozoite stage (Kyes, Pinches et al. 2000). No other *var* transcripts were detectable by Northern blot leading to the supposition that control of *var* gene expression is maintained at the level of transcription initiation. This contrasts with



data whereby reverse transcriptase and polymerase chain reaction (RT-PCR) has been employed as a means of detecting mRNA transcripts within a single infected erythrocyte. Results from these experiments would suggest that multiple *var* transcripts are present in ring stages with one dominant transcript present by the trophozoites stage (Chen, Fernandez et al. 1998; Scherf, Hernandez-Rivas et al. 1998). These results imply a post-transcriptional mechanism of control. It is interesting to note that RT-PCR picks up transcription of sporozoite and gametocyte stage-specific genes during the asexual cycle as well as multiple *var* transcripts. Perhaps then, RT-PCR is too sensitive a tool in detecting short transcripts and as a result is unable to distinguish between background low levels of transcription and meaningful specific activation.

Interestingly repression of *var* gene transcription during intraerythrocytic development of the parasite occurs at different times depending on the location of the *var* gene. Inhibition of subtelomeric *var* gene transcription occurred between 4 and 8 hours before that of centrally located *vars*. This illustrates that the structural differences of the three types of promoter reflect functional and regulatory differences in transcriptional repression (Voss, Kaestli et al. 2003). Whether these differences in control reflect differences in function and virulence with regards the different subsets of *var* gene remains to be seen. It is interesting to note however that *var* genes located subtelomerically are subject to frequent ectopic recombinant events compared to the more stable internally located *var* genes (Freitas-Junior, Bottius et al. 2000). Also of note is the finding that upsA-associated *var* genes, i.e. those situated subtelomerically, were more commonly expressed by parasites selected to express a phenotype (high frequency of recognition) similar to that found

in parasites isolated from cases of severe malaria (Jensen, Magistrado et al. 2004). This is consistent with the observation that *var* genes expressed from cases of severe malaria in Brazil, were more likely to have less cysteines present within the DBL1 $\alpha$  domain, a feature more common in *var* genes associated with the upsA promoter, (Kirchgatter and Portillo Hdel 2002), and also from work describing the expression of longer *PfEMP1* molecules being more common in parasites from cases of severe malaria (Bian, Wang et al. 1999).

Excitingly very recent data has given more weight to the differential regulation of expression of *var* genes from different genomic locations. A homologue to the yeast protein SIR2 (silent information regulator 2), known to have a role in silencing telomerically situated genes through alterations in chromatin compaction, called *PfSIR2*, was found to have a similar role in *P. falciparum*. Parasites with this gene knocked-out showed increased transcription of a sub-group of sub-telomerically situated *var* genes. It was also demonstrated that within the nucleus changes in spatial location of the telomeres was associated with active transcription of telomerically situated genes (Duraisingh, Voss et al. 2005; Freitas-Junior, Hernandez-Rivas et al. 2005). This has been demonstrated in trypanosomes where movement of a gene into a specific nuclear compartment allowed full transcription of this locus to the exclusion of all others (Navarro and Gull 2001). Whether a similar mechanism exists in *P. falciparum* remains to be seen.

The presence of the previously mentioned 5' flanking regions, specific to the different *var* types (upsA, B and C) and strongly correlated with chromosomal location as described, implies that regulation of transcription does not rely on the

presence of generalised *var* gene-specific transcription factors but rather every *var* gene represents a single transcriptional unit capable of *in situ* activation (Deitsch, del Pinal et al. 1999; Voss, Thompson et al. 2000). Removal of the promoter from its chromosomal location abrogates the silencing of the corresponding *var* gene (Deitsch, del Pinal et al. 1999). This finding was extended by the observation that silencing of *var* gene transcription requires cooperative interaction between the specific promoter and conserved sequences within the *var* gene intron (Deitsch, Calderwood et al. 2001). This finding is especially interesting when considering the previously described conserved *var* pseudogene *3D7chr5var2*. It is known that this gene is missing a substantial portion of its intron and that it is constitutively transcribed in around 60% of field isolates as well as many laboratory-adapted isolates (Kyes, Christodoulou et al. 2003; Winter, Chen et al. 2003). It may be that this pseudogene has lost the ability to silence itself.

Despite this inherent capability for activation, *var* gene expression occurs in a mutually exclusive way. It was observed that neither the chromosomal location nor the DNA sequence altered when comparing those *var* genes in the active state with those silenced, not only implying transcriptional control but also epigenetic mechanisms at work. It would appear from work undertaken in other organisms, that histone modification including acetylation, phosphorylation and methylation plays an important role in epigenetic regulation of gene expression. It is of interest that the *P. falciparum* genome identified an extensive range of genes involved in chromatin assembly and histone modification (Aravind, Iyer et al. 2003). This is supported by data showing repression of subsets of *var* genes through inhibition of

histone acetylation: a function of the *Pf*SIR2 protein as described previously (Duraisingh, Voss et al. 2005; Freitas-Junior, Hernandez-Rivas et al. 2005).

Despite the data supporting a transcriptional and epigenetic level of gene control there is a growing body of thought postulating that post-transcriptional gene silencing (PTGS), may indeed be the predominant mechanism by which one *var* gene is ultimately selected for expression (Borst, Bitter et al. 1995). In *P. knowlesi*, the homologues of the *var* genes are called *SICAvar* and these share many of the same biochemical and variant properties of the *var* genes (al-Khedery, Barnwell et al. 1999). Many transcripts relating to the 3' end of *SICAvar* genes are detectable at both the ring and trophozoite stages of development. This observation led some groups to hypothesize that PTGS may be functioning in *P. knowlesi* perhaps through an RNAi-mediated route (Galinski and Corredor 2004). In support of this viewpoint, antisense spliced *SICAvar* RNA is present throughout the intraerythrocytic development of the parasite, which may represent the products of RNA dependent RNA polymerase functioning to create double stranded RNA as would occur in a functioning RNAi system (Denli and Hannon 2003). Furthermore *Plasmodia* spp. have recently been shown to possess the ability to process double-stranded RNA into small 25 nucleotide RNA packets, an intrinsic quality of RNAi mediated PTGS (Malhotra, Dasaradhi et al. 2002). Despite this result however, it seems unlikely this is the predominant mechanism operating in *P. falciparum* as the necessary genes are not identifiable within the *P. falciparum* genome {C. Newbold *personal communication*}.

### **Other *P. falciparum* polymorphic gene families**

The publication of the *P. falciparum* genome in 2002 highlighted the significant proportion of the genome devoted to multigene families, suggesting the importance of immune evasion to the parasite (Gardner, Hall et al. 2002). In addition to the *var* genes, are the families *rif*, *stevor*, *pf60* and *surf* (Weber 1988; Carcy, Bonnefoy et al. 1994; Cheng, Cloonan et al. 1998; Winter, Kawai et al. 2005).

The *rif* (repetitive interspersed family) family of genes was discovered in 1988 when interspersed repetitive DNA was isolated from a genomic library of *P. falciparum*. Northern blot analysis identified that this sequence was transcribed during late asexual growth stages within the erythrocyte, however no protein translation initiation codon could be located and protein products were not detected (Weber 1988). Subsequent analysis of the *P. falciparum* genome revealed not only that there were an extremely high copy number of these genes, 149 in the 3D7 genome, but also that 300bp upstream of each *rif* gene was a sequence encoding a translational start and a putative signal sequence (Gardner, Hall et al. 2002). The *rif* genes have a two exon structure and are located subtelomerically on all 14 chromosomes. The first exon encodes a signal peptide and the second, a putative transmembrane region and a polymorphic extracellular domain, which includes conserved cysteine residues and a highly variable region (Cheng, Cloonan et al. 1998). A further transmembrane region follows the extracellular domain with a short, conserved cytoplasmic tail. The proteins encoded by these genes (RIFINS) are expressed in the late ring or early trophozoite stage and are located on the surface of the infected red blood cell temporally associated with the expressed *var* gene product (Fernandez, Hommel et al. 1999; Kyes, Rowe et al. 1999; Kyes, Pinches et al. 2000). Each

RIFIN protein is around 30-40kDa in size, undergoes antigenic variation and is a target for naturally occurring antibodies in sera from adults in endemic areas (Abdel-Latif, Khattab et al. 2002). The precise function of the expressed *rif* genes is presently unknown however their co-localisation on the surface of infected red blood cells with *Pf*EMP1 as well as their close proximity with *var* genes at the telomeres, have led to speculation that they are involved in either trafficking of *Pf*EMP1 or regulation of expression of *var* genes.

A third multigene family, *stevor* (subtelomeric variable open-reading frame), closely related in terms of structure and chromosomal location, to the *rif* gene family was identified in 1990 (Limpaiboon, Taylor et al. 1990). Each *stevor* gene encodes a 30-40kDa protein and like the *rif* genes have a two exon structure. A short first exon similar in both families encodes a start codon and predicted signal sequence. The second exon is shorter than that found in the *rif* genes, 1kb compared to 1.3kb, and encodes a hypervariable area flanked by transmembrane domains (Cheng, Cloonan et al. 1998). The genome-sequencing project highlighted that all 28 *stevor* genes present within the 3D7 genome share this domain pattern. The *rif* genes display a far less uniform structure as a family (Gardner, Hall et al. 2002). As with the *rif* genes, *stevor* genes are located subtelomerically on all 14 chromosomes. Multiple *stevor* transcripts have been detected within a single parasite, with peak transcription occurring at around 28 hours post invasion of the red cell, coinciding with the mid-trophozoite stage, later than expression of both the *rif* and *var* gene families (Kaviratne, Khan et al. 2002). It is unclear presently, whether the presence of multiple transcripts is correlated with the expression of more than one expressed STEVOR product. Immunofluorescence experiments using monoclonal and

polyclonal antibodies raised against STEVOR proteins have demonstrated that the expressed protein does not localise to the red cell membrane but rather migrates to the Maurer's clefts (Kaviratne, Khan et al. 2002). Maurer's clefts are membranous structures situated just beneath the red cell membrane, which have been implicated in the transport of parasite products to the surface of the red cell. At the time of STEVOR expression, late trophozoites and schizonts are already sequestered and have expressed on their surface both *Pf*EMP1 and RIFIN proteins. It would seem thus that the function of STEVOR is clearly distinct from that of the RIFIN proteins despite being extremely similar gene families in terms of structure and location within the genome. More recently expression of STEVOR proteins has been observed in sporozoites and gametocytes. In gametocytes, stevor transcripts appear transiently early in development but STEVOR proteins persist for several days and are transported out of the parasite, travel through the host cell cytoplasm, and localize to the erythrocyte plasma membrane. In contrast to asexual parasites, gametocytes move STEVOR to the periphery via a trafficking pathway independent of Maurer's clefts. In sporozoites, STEVOR proteins appear dispersed throughout the cytoplasm in vesicle-like structures. It is likely that STEVOR variants perform different functions in each stage of the parasites life cycle in which they occur (McRobert, Preiser et al. 2004).

The *Pf60* family, which shares a high degree of homology with the second exon of the *var* genes, is composed of approximately 90 copies per haploid genome (Carcy, Bonnefoy et al. 1994). It is clear from the genome project that the majority of these genes are pseudogenes as they contain frameshifts or internal stop codons (Gardner, Hall et al. 2002). However one member of this family has been fully characterised and unusually a mature protein is expressed through the presence of additional 5'

exons allowing a translational frameshift (Bischoff, Guillotte et al. 2000). The mature protein is located in the nucleus. How many other mature proteins are similarly expressed or whether or not this mechanism of translational frameshifting is employed in the expression of other *P. falciparum* proteins is unknown.

The most recently discovered family of polymorphic antigens are the SURFINS (Winter, Kawai et al. 2005). At present 10 copies of this family of genes, denoted *surf*, have been described within the 3D7 genome, and located on five chromosomes at or near the telomere. Structural and sequence similarity has been described to *PfEMP1* as well as to other surface-exposed proteins of different *Plasmodium* species, *PvSTP1*, *PkSICAvar*, *PvVIR*, *Pf332* (Winter, Kawai et al. 2005).

### **Antigenic variation**

Organisms that rely on an insect vector to complete their life cycle or exist in an ecological niche where transmission can be sporadic or seasonal have evolved tactics to ensure their survival through maintenance of infection within the mammalian host. These include *Trypanosoma* spp., *Borelia* spp. and *Neisseria* spp as well as *Plasmodia* spp. These pathogens all undergo clonal antigenic variation. Surface molecules exposed to the host immune response, are encoded in the genome as multi-copy gene families. Antigenic variation occurs by the successive switching of members of these families, each variant having little or no immunological cross-reactivity. The precise signals involved in promoting a switch and the molecular mechanisms that underpin antigenic variation are complex and difficult to disentangle.



Chronic infection is characterised by successive peaks of parasitaemia associated with the development of antibodies specific to each peak. As discussed each *P. falciparum* parasite has a *var* gene repertoire of around 60 and *in vitro* cloning studies show that switching away from a parental variant type can occur at a rate of up to 2% per generation in the absence of immune pressure (Roberts, Craig et al. 1992). If this switching rate occurred to the same degree *in vivo*, the parasite would rapidly exhaust its variant repertoire. Instead, as well as new repertoires of variant antigens becoming available for selection through telomeric mitotic recombination events, it is likely that there is a wide range of switch rates, with expression of some variants occurring more frequently than others. This could potentially result in a hierarchy of expression determined by different switch rates. This is certainly the case with *P. fragile* infections in the toque monkey (Handunnetti, Mendis et al. 1987), and has been predicted from modelling work to occur in *P. falciparum* (Molineaux and Dietz 1999; Molineaux, Diebner et al. 2001). Indeed, mosquito-induced experimental infections in human volunteers highlighted a switching rate away from the initial dominant expressed *var* gene of 16% per generation, much higher than the 2% described *in vitro*. Subsequent switching occurred at a slower rate (Peters, Fowler et al. 2002). The composition and frequency of *var* transcripts in the parasites isolated from the human volunteers were dramatically different from the parent parasite used to infect the mosquito. There would thus seem to be a complete changeover in *var* gene expression after parasites pass through the mosquito vector. The pattern of subsequent transcription, at least early on in the infection seemed to be an intrinsic property of the set of *var* genes present within the infecting parasite as the same switch to an alternative expressed *var* was seen in more than one human volunteer (Peters, Fowler et al. 2002), although as this work

was done using PCR and no actual protein expression or changes in antigenic phenotype were identified, it is possible that lack of specificity may have led to multiple *var* transcripts being detected thus caution in interpretation of these data is advised. Similarly, *in vitro*, switching rates were variable within a panel of parasite clones and between parasites expressing the same variant, switching rates were identical (Horrocks, Pinches et al. 2004), implying the rate of switching is an intrinsic property of individual *var* genes.

The signals controlling regulation and initiation of switching within the parasite are as yet unknown. There is some evidence suggesting communication between the host and the parasite can occur. In 1973 Brown and colleagues immunised rhesus monkeys with *P. knowlesi* bearing a particular phenotype resulting in the production of high titres of variant-specific antibodies (Brown 1973). They then went on to infect both immunised and naïve monkeys with 10, 100 or 1000 *P. knowlesi* parasites expressing the same SICA antigen to which the immunised monkeys had already been sensitised. The resulting infections in both the immunised and naïve monkeys were almost identical in terms of the pre-patent period and subsequent parasitaemia. However the infections in the sensitised monkeys contained parasites expressing a different SICA antigen on the surface of the erythrocyte. It was argued that the difference in phenotype could not have occurred through selection of subpopulations of parasites expressing different variants because of the limited inocula used and the kinetics of appearance of a patent parasitaemia. It was concluded that homologous anti-variant antibody acted as a signal, from the host to the parasite, inducing switching. This hypothesis would suggest that switching away from a given variant would not occur until after the appearance of a specific anti-

variant antibody, so protecting the variant repertoire from rapid exhaustion. However in the recent study examining switching in naïve human volunteers, there was evidence of rapid switching away from the parental dominant expressed *var* gene on establishment of initial blood stage infection (Peters, Fowler et al. 2002). Furthermore attempts to show evidence of a similar phenomenon *in vitro*, using hyper immune sera from adults resident in Kilifi, Kenya and a monoclonal antibody directed against a specific *PfEMP1*, A4 *var*, proved inconclusive {P.Horrocks *personal communication*}. Thus perhaps the initial interpretation was wrong, perhaps antibody subclass is important or it may be that signals in addition to specific antibody are necessary to induce switching.

Further evidence supporting host-parasite signalling comes from data illustrating the role of the spleen in determining the surface characteristics of *P. knowlesi* infected erythrocytes. Passage of *P. knowlesi* parasites through splenectomised rhesus monkeys resulted in infected erythrocytes no longer displaying on their surface SICA variants. They were not agglutinated by immune sera or indeed specific anti-sera from monkeys previously infected with the homologous parasite. Surface-iodinated SICA antigens could no longer be immunoprecipitated from these populations of infected erythrocytes. Furthermore these SICA negative parasites were less lethal than the SICA positive variants suggesting a link between these surface variant antigens and virulence. If these SICA negative parasites were passaged again through a spleen-intact monkey the SICA antigens once again appeared on the surface of the infected red cell (Barnwell, Howard et al. 1982; Barnwell, Howard et al. 1983). These observations give further support to the premise that the *in vivo* environment may provide signals that influence the

expression and switching of variant antigens and consequently the phenotype of the infected red cells.

The erythrocyte is apparently the perfect ecological niche for the malaria parasite: it does not differentiate, has no internal protein trafficking or synthesis capability and does not possess any MHC molecules. As a result there is considerable speculation as to why the parasite has evolved mechanisms whereby parasite-derived proteins are transported to the surface of the infected red cell and expressed on its surface in full view of the host's immune response. As a consequence of this action considerable effort is expended by the parasite to evade recognition by the host through antigenic variation. It must be crucial to the parasite that these proteins are inserted into the membrane and thus they must perform a critical function. The fact that cytoadherence to endothelial cells removes mature forms of the intraerythrocytic parasite from the circulation, so preventing removal of pRBCs by the spleen, would seem a compelling reason to insert these proteins into the surface membrane. However, variant surface antigens have been detected in all species of malaria and yet only a minority of these, as far as we know, display any cytoadherent properties. This would thus argue that perhaps expression of these surface-bound variant proteins predates, in evolutionary terms, cytoadherence.

Another possible reason for the evolution of antigenic variation is that perhaps it is in the parasite's favour to expose themselves to the host in order to allow control of parasite numbers. In naïve humans, the initial growth rate of *P. falciparum* is extremely high and if it were left to continue unabated would result in complete erythrocyte destruction and death of the host before transmission occurred (Cheng,

Lawrence et al. 1997). Thus by the inability to cytoadhere, it is conceivable that in the absence of variant antigen expression, parasites would multiply and quickly overcome the host, thus abrogating their chance of transmission (Saul 1999). However, data does not support this hypothesis. Lines of both *P. falciparum* and *P. knowlesi* which are temporarily unable to express variant antigens on the surface of red cells are less virulent and achieve lower parasitaemias than their wild-type counterparts (Barnwell, Howard et al. 1983; Langreth and Peterson 1985).

It is clear from experimental evidence, that parasites that don't express on their surface variant proteins are both less virulent and more immunogenic (Barnwell, Howard et al. 1983; Langreth and Peterson 1985). *PfEMP1* has been shown to inhibit the maturation and activation of dendritic cells (Urban, Ferguson et al. 1999), so leading to an impaired host immune response and thus potentially prolonging the life and, as a result, transmissibility of the parasite: a possible alternative reason for the development of antigenic variation as a consequence of displaying these surface-exposed proteins.

## ***PfEMP1* and pathogenesis**

### **Cytoadherence**

Unique amongst human malarias, *P. falciparum* can avoid removal by the spleen by sequestering its mature forms within the vasculature. Post-mortem examinations of people who have died from *P. falciparum* infection show accumulation of infected erythrocytes within the small vessels of many tissues (MacPherson, Warrell et al. 1985; Turner, Morrison et al. 1994). This phenomenon is likely to be important by contributing to high total body parasitaemia and focusing pathology in distinct sites.

As an example, several post-mortem studies have demonstrated greater infected erythrocyte sequestration in the brain compared with other organs in individuals dying of cerebral malaria as defined clinically (MacPherson, Warrell et al. 1985; Oo, Aikawa et al. 1987).

The observed sequestration of infected erythrocytes and the possible associations with clinical presentation of *P. falciparum* infection has led to intense interest in the molecular mechanisms of cytoadherence and the ligands involved both host and parasite derived. Although it is mainly the more mature forms of the intra-erythrocytic *P. falciparum* stages that sequester this is not exclusively so. Recent evidence suggests that ring-stages, as early as 4 hours post-erythrocyte invasion were capable of binding to several cultured cell types *in vitro*, albeit to a much lesser degree than trophozoites (Pouvelle, Buffet et al. 2000). In addition there is some histopathological evidence in support of this although whether the accumulation of ring-stages seen is due to specific receptor binding or simply reflects the passive accumulation of early parasitised red cells is unknown (Silamut, Phu et al. 1999). Within the placenta 90% of parasitised erythrocytes are of the mature trophozoite and schizont stage with the proportion of ring-forms in the placental and peripheral circulations not significantly different, results consistent with a prominent role for mature asexual parasites in placental malaria (Beeson, Amin et al. 2002).

It is clear that infected erythrocytes display multiple adhesive properties and that these vary between clones (Roberts, Craig et al. 1992) (Biggs, Anders et al. 1992). It is also clear that, to a large extent, *PfEMP1* is responsible for the variability of the adhesive characteristics of different parasite clones. In vitro studies have identified

several cell-surface molecules as potential receptors for infected erythrocyte binding, including thrombospondin (TSP), CD36, intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule, E-selectin, chondroitin sulphate A (CSA), CD31, complement receptor-1 (CR1), heparin sulphate and hyaluronic acid (HA) (Roberts, Sherwood et al. 1985; Barnwell, Asch et al. 1989; Berendt, Simmons et al. 1989; Ockenhouse, Tegoshi et al. 1992; Rogerson, Chaiyaroj et al. 1995; Rowe, Moulds et al. 1997; Treutiger, Heddini et al. 1997; Beeson, Rogerson et al. 2000; Vogt, Barragan et al. 2003). In addition to adhering to endothelial cells and syncytiotrophoblasts, mature-stage pRBCs can also adhere to non-infected erythrocytes (Handunnetti, David et al. 1989), forming rosettes, and to other infected erythrocytes, forming clumps (with platelets) or autoagglutinates (Roberts, Pain et al. 2000; Pain, Ferguson et al. 2001).

Establishing a direct cause and effect relationship between sequestration and severe malaria has proven difficult. This in part is likely to be due to the heterogeneity of pathogenic processes that can lead to the end point of severe disease, as indicated in clinical studies, (Marsh, Forster et al. 1995), and recently supported by detailed histological studies in Malawian children where out of 31 patients with clinically defined cerebral malaria, 7 had alternative non-malaria causes of death identified at post-mortem (Taylor, Fu et al. 2004). During pregnancy, infected erythrocytes typically sequester in the placenta, and maternal malaria is associated with intra-uterine growth retardation and premature delivery resulting in excess neonatal mortality (Steketee, Nahlen et al. 2001; Shulman and Dorman 2003). Maternal health also suffers through the development of maternal anaemia and the resultant increased likelihood of maternal death (Shulman and Dorman 2003).

The consequences of sequestration to the host are thus variable and can be severe. Although the link between sequestration and clinical disease in humans remains indirect, in vasculature where this phenomenon occurs a degree of obstruction to blood flow will occur. This could lead to hypoxia, reduction of metabolite exchange and the release of inflammatory mediators. In the brain it could contribute directly to cerebral oedema and raised intracranial pressure (Newton, Peshu et al. 1994).

### **Host receptors**

Many host receptors have been associated with a role in the adhesion of infected erythrocytes but individually their specific role in pathogenesis is unclear. Organ-specific sequestration undoubtedly involves multiple receptors and the different combinations afforded by expression of particular *PfEMP1* variants with the possible combinations of host receptors may determine the principal site of sequestration and thus the resultant clinical features.

### **CD36**

CD36 is an 88-kDa glycoprotein found on the surface of platelets, monocytes, dendritic cells and microvascular endothelial cells. In a histo-pathological study of post-mortem material from individuals dying of cerebral malaria, very little CD36 expression was found on cerebral endothelium but it was present ubiquitously on lung, liver, kidney, skin and muscle vasculature (Turner, Morrison et al. 1994; Turner, Ly et al. 1998).



*In vitro*, mature parasitised erythrocytes can bind to CD36 (Barnwell, Asch et al. 1989; Oquendo, Hundt et al. 1989). The majority of clinical isolates causing disease in non-pregnant individuals also bind to CD36 (Ockenhouse, Klotz et al. 1991; Newbold, Warn et al. 1997; Rogerson, Tembenu et al. 1999). The relationship between CD36-binding and pathogenesis however, is not clear. In a large study in Kenya the ability of 200 isolates to bind to a range of endothelial cell receptors including CD36, was assessed quantitatively (Newbold, Warn et al. 1997). There was no difference in CD36 binding between isolates from well, asymptotically parasitised children and those with cerebral malaria. There was, however significantly higher binding to CD36 in children with non-severe symptomatic malaria. These results are in accord with a study from Malawi where an inverse relationship was found between binding to CD36 and disease severity (Rogerson, Tembenu et al. 1999).

In support of this, in malarious areas of the Western Pacific, Southeast Asian ovalocytosis (SAO) is a genetic trait, which occurs at high frequencies and provides some protection against severe disease especially cerebral malaria (Allen, O'Donnell et al. 1999). SAO is caused by a deletion of 27 base pairs of the erythrocyte membrane band 3 gene on chromosome 17 (Jarolim, Palek et al. 1991). It has been reported that under flow conditions, SAO infected red cells bind at significantly higher levels to CD36 compared to non-SAO infected red cells (Cortes, Benet et al. 2004). This suggests that the protection afforded to SAO individuals against cerebral malaria may be due to an altered distribution of sequestered parasite mass, with infected red cells preferentially binding to CD36 and thus avoiding cerebral vasculature.

Two studies examining the effects of a CD36 mutation, present at high frequencies in some African populations and resulting in a deficiency of CD36, on susceptibility to or protection from clinical malaria, have found conflicting results. In one study an association with susceptibility to severe, in particular cerebral malaria was described, (Aitman, Cooper et al. 2000), and in the other association with protection against some forms of severe malarial disease was found (Pain, Urban et al. 2001).

### ICAM-1

ICAM-1 is member of the immunoglobulin super-family and supports parasite adhesion both *in vitro* and *in vivo* (Berendt, Simmons et al. 1989; Ho, Hickey et al. 2000). A good case can be made for ICAM-1 being a key host receptor in the causation of cerebral malaria. It is widely expressed on cerebral endothelium, is upregulated in the presence of pro-inflammatory cytokines such as tumour necrosis factor (TNF) and in a histo-pathological study was co-localised with infected erythrocytes in the brains of patients dying from cerebral malaria (Turner, Morrison et al. 1994).

Looking quantitatively at binding to ICAM-1 and relating this to disease severity, the same study previously described found in Kenya a positive association with binding to ICAM-1 *in vitro* and severe malaria (Newbold, Warn et al. 1997). However in Malawi the opposite association was observed (Rogerson, Tembenu et al. 1999).

Sequencing of ICAM-1 has revealed a high frequency mutation, termed ICAM-1<sup>kilifi</sup>, present in individuals from East and West Africa but absent in Europeans

(Fernandez-Reyes, Craig et al. 1997). Using a case-control framework, the effect of this mutation on susceptibility to severe malarial disease was examined amongst children resident in a malaria-endemic area of Kenya. Homozygotes for this mutation were found to be at a two-fold higher risk of cerebral malaria than controls. In West Africa, one study showed the opposite association, (Kun, Klabunde et al. 1999), with yet another study demonstrating no association (Bellamy, Kwiatkowski et al. 1998). It is possible that this mutation was selected for another reason within these populations. It is of note that a positive association with protection from non-malarial infectious disease in Kenya has been demonstrated with ICAM-1<sup>kilifi</sup> (Jenkins, Mwangi et al. 2005)

## CSA

Parasite adhesion to CSA has been implicated as a key pathological step in the development of placental malaria (Fried and Duffy 1996; Beeson, Brown et al. 1999). Several studies have shown that parasites accumulated in the placenta typically adhere to CSA and *in vitro*, infected erythrocytes adhere to placental vascular tissue in a CSA-dependent manner (Fried and Duffy 1996; Beeson, Brown et al. 1999; Gysin, Pouvelle et al. 1999). This contrasts with parasites isolated from children or non-pregnant adults, which show little or no adherence to CSA (Fried and Duffy 1996; Beeson, Brown et al. 1999) (Chaiyaroj, Angkasekwinai et al. 1996; Rogerson, Tembenu et al. 1999). Placental isolates show little binding to receptors such as CD36 or ICAM-1, and if parasites are selected for binding to CSA, they lose the ability to bind to these receptors (Rogerson, Chaiyaroj et al. 1995; Gamain, Smith et al. 2001). However parasites isolated from the peripheral blood of pregnant women show binding to both CSA and CD36 suggesting these circulating ring-stage

parasites have arisen from both placentally-bound parasites and parasites sequestered elsewhere in the vasculature (Fried and Duffy 1996; Beeson, Brown et al. 1999). Interestingly there was no binding to ICAM-1 amongst parasites isolated from the placenta or peripheral blood of pregnant women, a finding in stark contrast to the binding patterns seen in parasites infecting children. ICAM-1 is present on the surface of syncytiotrophoblasts as well as being ubiquitous throughout the vascular system, it may be that immune responses against ICAM-1 binding parasite strains acquired during previous infections may be the reason behind this finding (Beeson, Brown et al. 1999).

### **Rosetting**

Rosetting, involving infected and uninfected erythrocytes, is observed with some, but not all parasite isolates. It appears to involve several erythrocyte ligands including heparan sulphate (HS), complement receptor 1 (CR1) and blood group antigens A and B (Rowe, Moulds et al. 1997; Chen, Barragan et al. 1998; Barragan, Kremsner et al. 2000).

Several, (Carlson, Helmby et al. 1990; Rowe, Obeiro et al. 1995), but not all, (al-Yaman, Genton et al. 1995), studies have demonstrated an association between the rosetting phenotype and disease severity in that parasites displaying the rosetting phenotype occur more often in children with severe disease. It has also been shown, again in some but not all studies, that antibodies that disrupt rosettes are less common in individuals with clinical disease (Carlson, Helmby et al. 1990; Reeder, Davern et al. 1997). Further support for an association between rosetting and disease

severity comes from recent work from Papua New Guinea. As mentioned CR1 has been shown to mediate the formation of rosettes and erythrocytes displaying CR1 deficiency show reduced rosetting (Rowe, Moulds et al. 1997). In Melanesian populations CR1 deficiency is common and in Papua New Guinea this deficiency was associated with protection from severe malaria (Cockburn, Mackinnon et al. 2004). Furthermore, indirect evidence linking rosetting to disease severity was established when it was shown that individuals of blood group A antigen are more likely to suffer severe malarial disease than those with any combinations involving blood groups B or O (Fischer and Boone 1998). Blood group A antigen was found to mediate rosetting in both laboratory-adapted and wild isolates (Carlson and Wahlgren 1992; Udomsangpetch, Todd et al. 1993).

### **Immunomodulation**

Dendritic cells represent a vital link between the innate and adaptive immune response to infection, and are responsible for presenting antigen, activating naïve T-cells and enhancing antibody production (Liu 2001). Immature dendritic cells are highly phagocytic, internalising antigens by various pathways including those involving CD36 (Albert, Pearce et al. 1998). Antigen uptake then induces dendritic cell maturation, characterised by increased expression of MHC class II molecules, costimulatory molecules (CD40, CD80, CD86) antigen processing and presentation, and secretion of cytokines that activate effector immune cells.

Parasitised erythrocytes that bind to monocyte-derived dendritic cells via CD36 modulate the maturation of dendritic cells *in vitro*. Human dendritic cells interacting with erythrocytes infected with parasites selected for CD36 binding did

not mature on stimulation with lipopolysaccharide (LPS) or TNF (Urban, Ferguson et al. 1999; Urban, Willcox et al. 2001). This inhibition of maturation of dendritic cell function was dependent on the affinity of the infected erythrocytes for CD36 (Urban, Ferguson et al. 1999). In accordance with this, the percentage of circulating mature dendritic cells in children with malaria, both mild and severe, was significantly reduced compared to healthy aparasitaemic children (Urban, Mwangi et al. 2001)

### ***Pf*EMP1 as the adhesive parasite ligand**

Adhesion to many endothelial cell receptors has been attributed to *Pf*EMP1. These include CD36, ICAM-1, HS, CSA and CR1 (Baruch, Ma et al. 1997; Rowe, Moulds et al. 1997; Chen, Barragan et al. 1998; Reeder, Cowman et al. 1999; Smith, Craig et al. 2000). Infected erythrocytes are able to bind to a range of host receptors with each parasite having the ability to bind to a subset of them only.

The sequencing of the *var* genes has enabled the mapping of specific receptor binding sites to motifs within *Pf*EMP1. There are binding sites for several molecules at the N-terminal head of the extracellular domain of *Pf*EMP1. *In vitro* data identified that three *Pf*EMP1 domains, DBL1 $\alpha$ , CIDR1 $\alpha$  and DBL2 $\delta$  mediated binding independently to a number of host receptors (Chen, Heddini et al. 2000). These interactions included the identification of DBL1 $\alpha$  as the parasite ligand binding to CR1 thus mediating rosetting and also CIDR1 $\alpha$  as the domain mediating binding to CD36. It is important to stress that not all expressed *var* genes have the same binding properties and domain-specific binding varies considerably between *vars*.

Binding of infected erythrocytes to CD36 is especially interesting as it is a feature of many parasite isolates (Ockenhouse, Ho et al. 1991; Newbold, Warn et al. 1997; Rogerson, Tembenu et al. 1999). Antibodies raised against a recombinant protein corresponding to a portion of the CIDR1 $\alpha$  domain of two *var* genes present within the Malayan Camp parasite isolate, blocked adherence of this strain to immobilised CD36 (Baruch, Pasloske et al. 1995). Furthermore these antibodies immunoprecipitated the same fragment of CIDR1 $\alpha$  as purified CD36 (Baruch, Gormely et al. 1996) and, when anti-CD36 monoclonal antibodies were shown to block adherence of parasitised erythrocytes to CD36 this occurred regardless of differences in the expressed *Pf*EMP1 protein (Barnwell, Asch et al. 1989; Hasler, Albrecht et al. 1993). This suggests that most variants of *Pf*EMP1 bind to the same region of CD36 and as such the CD36-binding domain of *Pf*EMP1 may have some conserved sequence and structure. Analysis of the sequences corresponding from diverse parasites to the CD36 binding region of *Pf*EMP1, located within the CIDR1 $\alpha$  domain, demonstrated that these sequences were not identical but did display some homology. This was most apparent by conservation of cysteine residues at either end of the fragment resulting in a highly conserved predicted three-dimensional structure (Baruch, Ma et al. 1997). It is thought that this structure is maintained regardless of differences in amino acid sequence and that it is the conformation of the protein fragment rather than the sequence itself that is essential for function.

Exceptions to CIDR1 $\alpha$  mediating binding to CD36 are CSA-binding parasite isolates. *Pf*EMP1 was discovered to be a parasite ligand mediating binding to CSA in 1999 (Reeder, Cowman et al. 1999). Selection of infected erythrocytes on CSA *in vitro*

leads to a loss of CD36 and ICAM-1 binding and the ability to form rosettes (Rogerson, Chaiyaroj et al. 1995; Gamain, Smith et al. 2001; Beeson and Brown 2004). As a consequence of binding to CSA via CIDR1 $\alpha$ , CSA and CD36-binding are mutually exclusive phenotypes (Gamain, Gratepanche et al. 2002). Selection of a laboratory isolate for binding to CSA initiated a switch in the *var* gene expressed coinciding with a change in antigenic type. A specific *var* gene was transcribed in these CSA-selected parasites, different to the dominant *var* transcribed in the parental non-selected clone (Reeder, Cowman et al. 1999). This *var* gene was called CS2. The direct association of this *Pf*EMP1 variant and CSA binding was demonstrated by almost complete reduction in adhesion in the presence of antibodies raised against some of the domains of this protein, specifically anti-DBL3 $\gamma$  and anti-CIDR1 $\alpha$  antibodies. Interestingly the inhibitory effect of anti-DBL3 $\gamma$  antibodies was specific to CSA binding but the anti-CIDR1 $\alpha$  antibodies also abrogated binding of other non-CSA selected parasite lines to CD36. In fact it has become clear that although the *Pf*EMP1 variants expressed on the surface of CSA-binding parasites contain CIDR1 $\alpha$  domains they do not bind to CD36 (Buffet, Gamain et al. 1999; Reeder, Cowman et al. 1999). This suggests that the DBL $\gamma$ -type and the CIDR1 $\alpha$  domains of parasites displaying binding to CSA may combine to form the CSA-binding region. Further work suggested that recombinant CIDR1 $\alpha$  domains on their own might be able to mediate binding to CSA in the absence of DBL $\gamma$ -type domains (Degen, Weiss et al. 2000). However, the expressed CIDR1 $\alpha$  protein in this particular case was isolated from a parasite line unable to bind CSA and so this adhesion characteristic is unlikely to represent a mechanism utilised *in vivo*.



Although binding to CSA has been largely attributed to the DBL $\gamma$  domains of *PfEMP1*, many expressed DBL $\gamma$  domains do not bind CSA (Gamain, Smith et al. 2004). There are varying amounts of homology and diversity in sequence amongst DBL $\gamma$  domains without any clear conserved areas among those domains that do bind CSA (Gamain, Smith et al. 2004). An analysis of 5 DBL $\gamma$  sequences from placental isolates revealed a homology ranging from 39 to 55% (Khattab, Kun et al. 2001).

The suggestion from serological studies, (Fried, Nosten et al. 1998), that a conserved ligand may be responsible for placental binding and may thus be amenable to targeting in a pregnancy-associated-malaria specific vaccine was given support by the finding that one *var* gene was the dominant transcript in many placental isolates as well as many isolates selected for adhesion to CSA (Duffy MF 2003; Salanti, Staalsoe et al. 2003). This gene, called *var2csa*, has an atypical structure lacking both DBL $\gamma$  and CIDR1 $\alpha$  domains instead possessing three DBLX-type and three DBL- $\epsilon$ -type domains (Salanti, Staalsoe et al. 2003). Until recently a CSA-binding domain had not been identified for this gene although substantial homology existed between the third DBL domain of *var2csa* and the minimal-binding region of the DBL $\gamma$  domain of another commonly expressed CSA-binding *var* gene, *FCR3varCSA* (Gamain, Smith et al. 2004). Recent work, however, has led to the identification of multiple CSA-binding domains within both 3D7 and A4 *var2csa* genes, including DBL2-X and DBL6-  $\epsilon$ . Of note though the third DBL domain, which as described showed homology to the CSA binding region of *FCR3varCSA*, did not bind to CSA (Gamain, Trimnell et al. 2005). Despite these encouraging results, the role of *var2csa* remains controversial. Analysis of expression of membrane proteins in placental and CSA-binding isolates showed that neither *var2csa* nor *FCR3varCSA*

were preferentially expressed on the surface of the infected erythrocyte (Fried, Wendler et al. 2004).

As described, another major receptor for infected erythrocytes is ICAM-1 (Berendt, Simmons et al. 1989). The binding site for ICAM-1 on *PfEMP1* has been mapped to the DBL2 $\beta$ -c2 region (Smith, Craig et al. 2000). This region shares only low homology with the same area in other ICAM-1 binding parasites, indeed showing the same degree of homology with DBL $\beta$  domains from non-ICAM-1 binding parasites (Smith, Subramanian et al. 2000). The domain CIDR1 $\alpha$  has exhibited weak affinity for ICAM-1 although the relative importance of this interaction *in vivo* remains to be seen (Chen, Heddini et al. 2000).

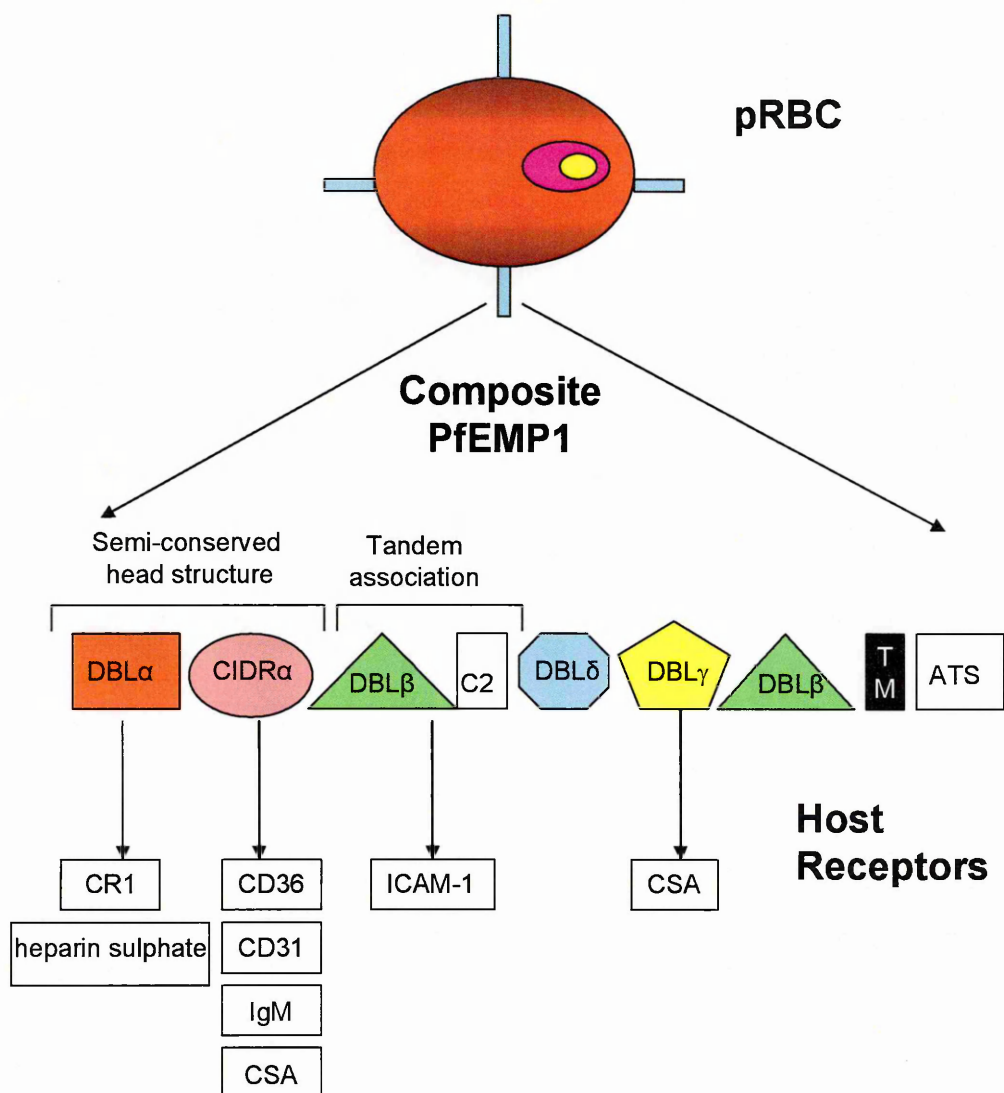
Rosetting has been linked to the DBL1 $\alpha$  domain (Rowe, Moulds et al. 1997; Chen, Barragan et al. 1998). A recombinant fusion protein expressing DBL1 $\alpha$  was found to adhere directly to normal erythrocytes, disrupt naturally formed rosettes and block rosette reformation. However, although the majority of *var* genes appear to possess a DBL $\alpha$ -type domain, 58 out of the 59 sequenced in *P. falciparum* genome project, not all parasite isolates form rosettes or adhere to the receptors involved. A summary of the known binding characteristics of *PfEMP1* is given in figure 1.7.

### **Other adhesive ligands**

Other molecules present on the surface of infected erythrocytes have been proposed to act as adhesive ligands. Using an anti-idiotypic antibody raised against a CD36 monoclonal antibody, a large molecular weight protein was identified and called sequestrin (Ockenhouse, Klotz et al. 1991). This protein is around 270-kDa in size,

is specific to parasite-infected erythrocytes and is surface-labelled with radioiodine. To date a direct interaction between sequestrin and CD36 has not been reported nor has this protein been implicated in adhesion to other receptors.

It has been thought that *clag9* (*cytoadherence-linked asexual gene 9*) may be involved either directly or indirectly, in the binding of infected red blood cells to CD36. Targeted gene disruption of *clag9* resulted in the loss of binding to CD36, and antibodies against *clag9* protein have been found to inhibit binding to CD36 (Trenholme, Gardiner et al. 2000). By profiling expression of *clag9* in both CD36 and CSA binding parasites, it has been shown that expression of this protein, comparable in both parasite lines, is limited to rhoptries and young ring-stage parasites but is not present in trophozoites, the phase in the *P. falciparum* growth cycle when parasite-infected red blood cells sequester (Chaorattanakawe, Davis et al. 2004). It may be then that *clag9* has an indirect role in adhesion perhaps in trafficking of PfEMP1 or other surface-expressed proteins.



**Figure 1.7**

**Schematic representation of the binding properties of the parasite-encoded RBC surface protein *PfEMP1*.**

The intracellular acidic terminal sequence (ATS) is highly conserved and anchors the protein to parasite-derived knobs on the RBC surface. The highly variable extracellular region is assembled from three blocks of sequence which share homology; DBL, CIDR and C2. A representation is illustrated. The arrows indicate the known binding specificities of each PfEMP1 region with the downstream effects illustrated. The binding specificities of domains vary among different var genes. Any single domain from a particular var gene may not bind to all of the receptors shown.

Peptides derived from parasite-modified band 3, an intrinsic red cell anion-transport protein, inhibited adhesion to CD36 (Crandall, Collins et al. 1993). Band 3 has also been mooted as the parasite adhesion molecule responsible for binding to thrombospondin (TSP) (Lucas and Sherman 1998). Involvement in binding to other receptors has not been described.

### **Is *Pf*EMP1 a major target for naturally acquired antibodies?**

As described before, antibodies directed against the surface of *P. falciparum*-infected erythrocytes measured either through agglutination or flow cytometry, may well be targeting *Pf*EMP1. The evidence existing currently, although compelling remains indirect. Considering agglutination assays against the infected erythrocyte surface, only sera from malaria-exposed individuals resulted in agglutination and only infected erythrocytes were agglutinated. Furthermore, both for agglutination and indirect surface immunofluorescence, positive results were only obtained from 16 hours post-erythrocyte invasion onwards, in other words only when the parasites were mature trophozoites or schizonts. Also both agglutination and surface immunofluorescence were abrogated by trypsin treatment of the infected erythrocytes but not by neuraminidase (Marsh, Sherwood et al. 1986). By using a differential staining technique, Newbold and colleagues also showed the agglutination reaction was variant-specific as described before (Newbold, Pinches et al. 1992). Many subsequent studies assessing the human agglutinating antibody response against the infected erythrocyte have also demonstrated the variant-specificity of this response (Forsyth, Philip et al. 1989; Iqbal, Perlmann et al. 1993; Reeder, Rogerson et al. 1994; Bull, Lowe et al. 1998). Thus antigens targeted by

agglutinating antibodies would seem to have properties known to be associated with *PfEMP1*.

Flow cytometry was introduced as a means of measuring antibody reactivity against the infected erythrocyte surface because it allowed for automated analysis of a large number of samples, both sera and parasites. By using a parasite line selected with a known deletion at the 5' end of chromosome nine, Piper and colleagues concluded that the target for antibodies directed at the infected erythrocyte surface measured by flow cytometry was *PfEMP1* (Piper, Roberts et al. 1999). Parasites missing a portion of chromosome nine had previously been shown to lack the ability to bind to CD36, they were not agglutinated by immune sera and a protein of a similar size to *PfEMP1* was not precipitated from the surface of erythrocytes infected with these parasites by immune sera (Day, Karamalis et al. 1993). From this it was concluded that these mutant parasites did not express *PfEMP1* on the surface of the erythrocyte. When antibody reactivity against the surface of the erythrocyte infected with the mutant parasite was compared to that of wild-type parasites, a marked reduction in fluorescence intensity was observed (Piper, Roberts et al. 1999). Similarly a different group showed that antibody responses measured by flow cytometry were stage-specific, occurring only in late trophozoite and early schizont stages and were variant-specific in that depletion from sera of antibodies recognising one particular variant did not reduce its ability to label other parasite variants (Staalsoe, Giha et al. 1999). In addition trypsinisation of infected erythrocytes resulted in reduced surface antibody binding by 96% (Williams and Newbold 2003). In a landmark paper in 1995, Smith and colleagues demonstrated elegantly that switches of *var* gene expression within cloned parasite lines correlated well changes in the specificity of

agglutination and also that flow cytometry using a monoclonal antibody (BC6) specific for one particular expressed *var* product, A4 *PfEMP1*, revealed a specificity of recognition which correlated closely with expression of A4 *var* (Smith, Chitnis et al. 1995).

Thus convincing if circumstantial evidence exists for antibodies in human immune sera recognising on the surface of *P. falciparum*-infected erythrocytes, *PfEMP1* by both agglutination and flow cytometry.

Very few studies have looked specifically at antibodies present in the sera of individuals in malaria-endemic areas against defined epitopes of *PfEMP1*. In 1998 Staalsø and colleagues described antibody responses, measured by ELISA, against three conserved peptides from a laboratory strain of *PfEMP1* (Staalso, Khalil et al. 1998). These peptides corresponded to the relatively conserved N-terminal sequence containing the binding site for CD36 and the C-terminal sequence making up the intracellular portion of the integral membrane protein. The reactivity against the peptides increased with age and was also significantly greater in those individuals with an asymptomatic infection compared to those with symptoms of acute malaria. This result led to the tentative conclusion that the presence of peptide-specific antibodies was associated with asymptomatic parasitaemia and therefore protection. However it must be noted that in this study those asymptotically parasitised were significantly older than those with symptoms.

A further study described antibody responses to recombinant DBL1 $\alpha$  domains from nine wild isolates from Gabon. These were compared to responses against three

conserved peptides from DBL1 $\alpha$  (Oguariri, Borrmann et al. 2001). Again there was evidence of acquisition of responses with age against all the DBL1 $\alpha$  domains and also higher reactivity against the variable, intact domains compared to the conserved peptides.

Less directly, anti-rosetting antibodies in individuals from two malaria-endemic areas with different transmission characteristics showed evidence of exposure-related acquisition with regards age and transmission (Barragan, Kremsner et al. 1998). Their precise target however is open to conjecture. Interestingly anti-rosetting activity and agglutination correlated poorly in this and another study, suggesting antibodies targeting diverse epitopes (Rogerson, Beck et al. 1996). Both sets of antibodies may be targeting different epitopes of the same antigen, *PfEMP1*, however again this remains purely speculative.

### ***PfEMP1* as a vaccine candidate**

As must be apparent, there are many problems with the feasibility of *PfEMP1* as a vaccine candidate, not least of which is its immense variability. However it is undoubtedly the case that these antigens are targets for naturally acquired protection and furthermore this family of proteins underpins much of the virulence of *P. falciparum* infections via cytoadherence, antigenic variation and immunomodulation as described and it is for these reasons that any prospective vaccine involving *PfEMP1* is potentially attractive.



The aim of a blood-stage vaccine will be to decrease mortality. This may happen by reducing the sequestered parasite biomass or possibly by extending the sub-clinical period, perhaps through increased splenic destruction of infected erythrocytes. Despite the immense variability of the nucleotide sequence, there is conservation in the overall domain architecture of this protein with evidence of functional constraints within individual domains. For example, there is, in the majority of cases, a semi-conserved head structure comprising DBL1 $\alpha$  and CIDR1 $\alpha$ , which mediate binding to a range of endothelial cell receptors (Chen, Hedding et al. 2000). In addition, the structuring of *var* genes into types and the tentative but inconclusive observation that sub-telomerically situated *var* genes form a distinct group possibly over-represented amongst cases of severe malaria has led to renewed interest in the possibility of a vaccine (Bian, Wang et al. 1999; Kirchgatter and Portillo 2002; Jensen, Magistrado et al. 2004).

There are a number of *PfEMP1*-based vaccines in varying stages of development. Using recombinant proteins corresponding to DBL1 $\alpha$  of ten parasite isolates, rats were immunised and the subsequent sera assessed for cross-reactivity (Ogari, Mattei et al. 2003). While these rat antibodies recognised synthetic peptides derived from conserved regions of DBL1 $\alpha$ , they failed to recognise the surface of the homologously infected erythrocyte. There are potential reasons for this failure of recognition, including the possibility that the fusion proteins do not reflect the correct conformation of the intact DBL1 $\alpha$  domain and thus the rat antibodies would predominantly be directed against linear as opposed to conformational epitopes. More encouraging recent work using a prime-boost immunisation regime resulted in rat antibodies, which not only recognised the surface of the homologously infected

erythrocyte, but also disrupted pre-formed rosettes (Chen, Pettersson et al. 2004). This method, involved initially recombinant *Semliki forest virus (SFV)*- mini-*var* constructs, designed to allow the proteins DBL1 $\alpha$ , CIDR1 $\alpha$  and DBL2 $\beta$  to be expressed extracellularly but anchored to the cell membrane thus allowing them to fold in a similar way as in the parasite, followed by a boost using a linear recombinant fusion protein expressing DBL1 $\alpha$ .

Possibly the most promising *Pf*EMP1 vaccine candidate with the exception of pregnancy-associated malaria vaccines, is CIDR1 $\alpha$ . Gamain and colleagues demonstrated the cross reactivity of antibody responses against this region using monoclonal antibodies prepared against the CD36-binding region of CIDR1 $\alpha$  from a laboratory strain of parasite. This antibody recognised 9 of 10 heterologous recombinant CIDR1 $\alpha$  proteins expressed on the surface of Chinese hamster ovary (CHO) cells (Gamain, Miller et al. 2001). In 2002 vaccination of Aotus monkeys with one copy of CIDR1 $\alpha$  from MC parasites protected them from homologous challenge with the otherwise lethal MC parasite line, but offered no protection against challenge with an alternative parasite line, FVO (Baruch, Gamain et al. 2002). Using a prime-boost regime, Gratepanche and colleagues established cross-reactivity of the antibody response elicited following vaccination of mice with three variants of CIDR1 $\alpha$  (Gratepanche, Gamain et al. 2003). The cross-reactive antibodies were measured using flow cytometry against CHO cells expressing heterologous CIDR1 $\alpha$  variants on their surface although whether or not these responses would lead to cross-reactivity when challenged against intact heterologously infected erythrocytes remains to be seen.

Pregnancy-associated malaria provides a syndrome-specific example of the feasibility of vaccination based on *PfEMP1*. Indirect observation suggests the placenta can affect *PfEMP1* expression by selecting those variants expressing CSA-binding characteristics ensuring sequestration within the placental vasculature. Of obvious interest is which *PfEMP1* proteins are expressed by placental binding isolates and do these reflect a more conserved sub-group of antigens more amenable to exploitation in a vaccine? Recently some encouraging results suggest that this may indeed be the case. Sera from pregnant women resident in Africa inhibit adhesion onto CSA of parasites taken from pregnant women in Asia and vice versa (Fried, Nosten et al. 1998) also antibody reactivity in sera from pregnant women correlates strongly between multiple CSA-binding parasites from diverse regions (Fried, Nosten et al. 1998; Khattab, Reinhardt et al. 2004) A genome-wide transcriptional screen has led to the identification of a distinct *var* gene upregulated in a CSA-binding parasite line (Salanti, Staalsoe et al. 2003). As mentioned previously, this *var* gene, *var2csa*, is not only present within the genome of most parasite isolates, (Rowe and Kyes 2004), but is expressed on the surface of erythrocytes infected with CSA-adherent parasite lines from diverse geographical areas and may be an important target of protective antibodies in pregnant women (Salanti, Dahlback et al. 2004).

As already outlined, there is currently no reliable surrogate *in vitro* correlate of protective immunity against *P. falciparum*. The studies in this thesis aim to measure heterologous or presumed cross-reactive immune responses amongst a cohort of individuals resident within two areas of differing transmission intensity on the coast

of Kenya. These responses were related to the presence of asymptomatic parasite carriage and any association with protection from subsequent clinical malaria was assessed. An attempt was made to identify the target of these responses on the surface of infected erythrocytes through expression of recombinant proteins corresponding to domains of A4 *Pf*EMP1.

# Chapter 2

## Materials and methods

### Study Site

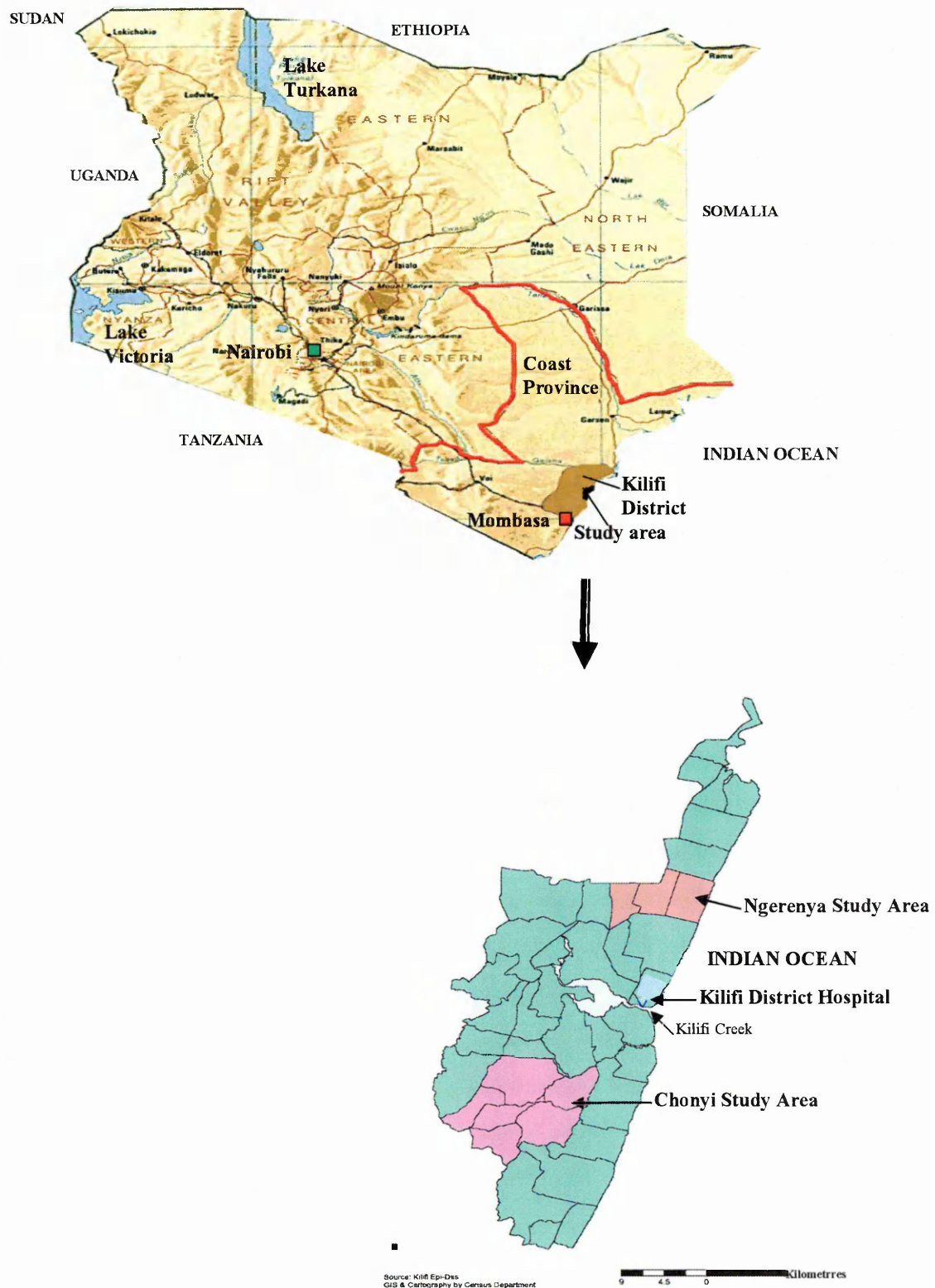
#### Description of areas under study

The studies outlined in this thesis were carried out at the Kenya Medical Research Institute Centre for Geographic Medicine Research Coast (KEMRI-CGMRC) situated within the grounds of Kilifi District Hospital in Kilifi, 50 km north of Mombasa on the coast of Kenya (figure 2.1). The hospital serves around 300,000 people living north and south of an ocean creek. Individuals whose immune responses were investigated throughout these studies were resident in two sites within Kilifi District within 20 km of each other, Chonyi and Ngerenya. Both areas experience perennial transmission with the most intense transmission occurring between May and July and December and January each year. Inhabitants of these areas are predominantly from the Mijikenda ethnic group and have similar beliefs and customs. Residents of Ngerenya have, on average 10 infective bites/person/year, (Mbogo, Snow et al. 1995) whereas residents of Chonyi have an estimated 50 bites/person/year (Mbogo, Mwangangi et al. 2003). The difference in transmission between the two areas is reflected in the parasite rate in each area and the proportion of individuals suffering an attack of clinical malaria during the period of follow up (figure 2.2 and figure 2.3). Within the individuals sampled in this study, in Chonyi in children aged 1 - 10 years, the parasite prevalence was 43.2% (95% CI 37.2 – 49.3%) and in Ngerenya over the same age range 23.6% (95% CI 18.9 – 28.9%). A study examining the clinical epidemiology of malaria under different transmission

conditions determined the incidence of clinical malaria in the two areas under study as being in Ngerenya 0.84 episodes/person/year and in Chonyi 0.55 episodes/person/year, evidence for lower incidence of malaria in the higher transmission area (incidence rate ratio - 0.66 (95% CI 0.61 – 0.72)  $p < 0.001$ ) (Mwangi, Ross et al. 2005). There was higher bed-net use amongst children less than 10 years in Ngerenya compared with that in Chonyi (69% vs. 6%) (Mwangi 2003). This is possibly because of a previous trial of bed-net usage undertaken in Ngerenya in the mid-1990s (Nevill, Some et al. 1996).

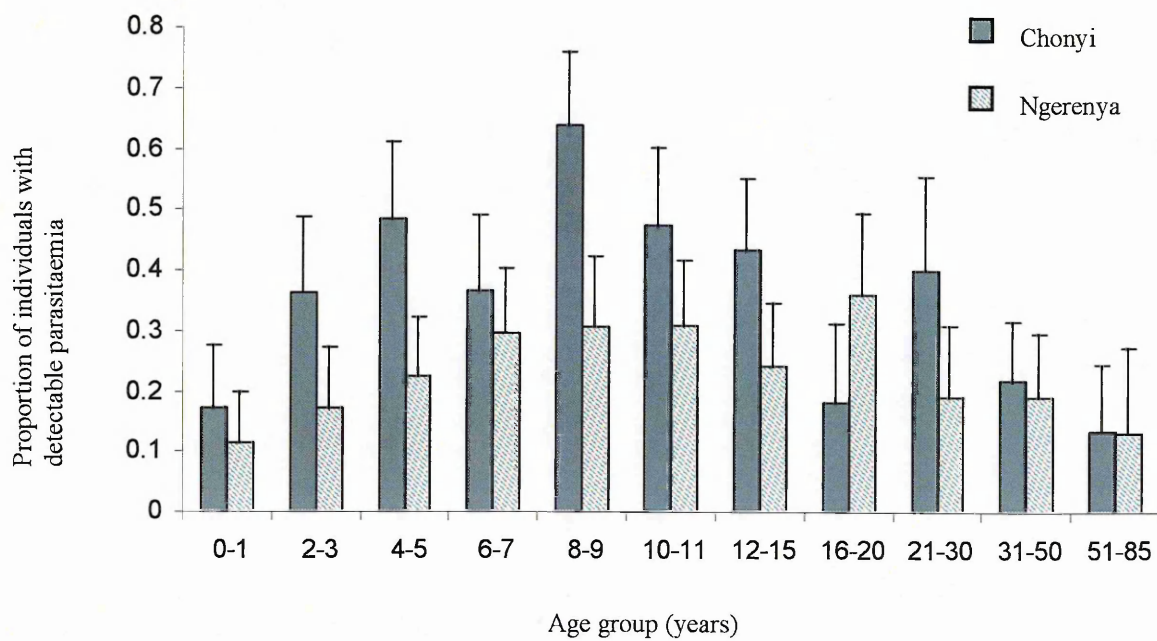
### **Sample collection**

Sera collection and active surveillance were conducted as part of a study examining the clinical epidemiology of malaria under differing transmission conditions (Mwangi 2003). In brief, serum was collected in October 2000 from individuals of all ages in both areas and the cohorts were followed for evidence of malaria by active and passive case detection for the subsequent 30 weeks. Malaria was defined as a febrile episode with an axillary temperature greater than 37.5°C and a parasitaemia of greater than 2500 parasites/ $\mu$ l except in children less than 1 year when fever (again  $>37.5^{\circ}\text{C}$ ) plus any parasitaemia counted as an episode. These have been determined to be optimal definitions for malaria among the different age groups in this area (Mwangi, Ross et al. 2005). A blood slide was prepared for every individual in the cohort at the time of serum collection in order to categorise him or her as parasite positive or negative by microscopy at the pre-transmission bleed. 20 non-malaria exposed control sera were collected from Oxford, United Kingdom.



**Figure 2.1**

**Map of study area**

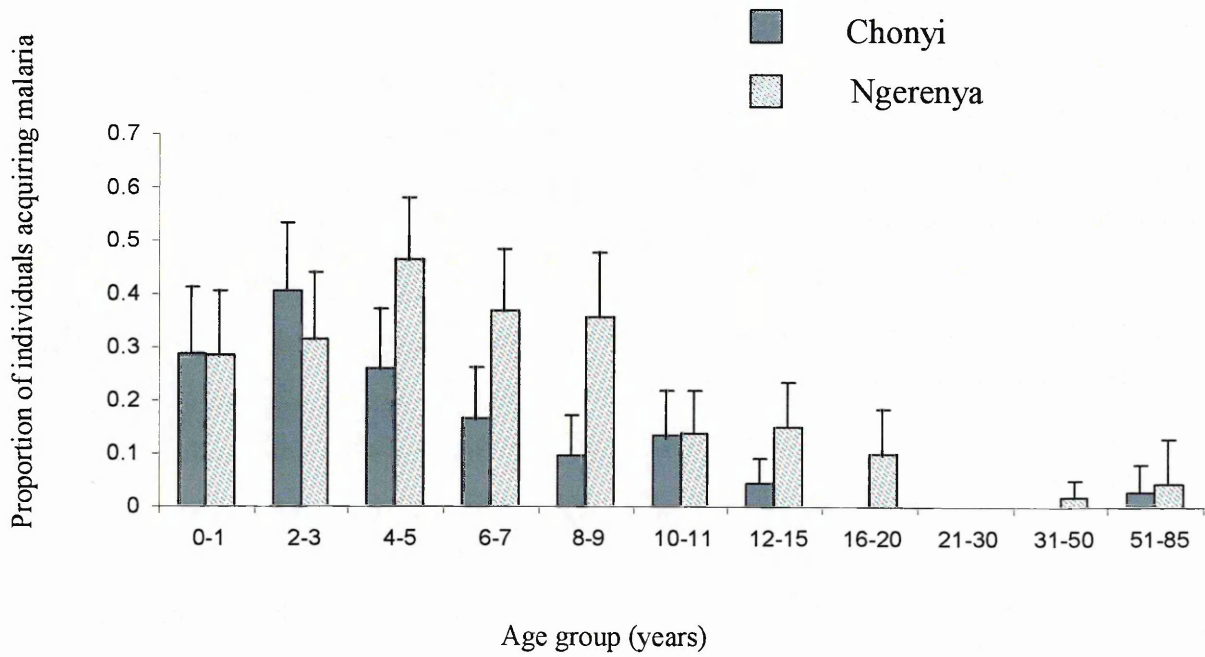


**Figure 2.2**

**Proportion of individuals in each age group in each area with microscopically detectable parasitaemia at serum collection.**

Serum collection occurred at the end of a low transmission period in October 2000.





**Figure 2.3**

**The proportion of individuals in each age group experiencing clinical malaria as defined in the text.**

Shown are overall proportions and upper 95% confidence intervals

## **Laboratory methods**

Each section of this thesis has a particular design and experimental outline, however a number of laboratory techniques are used throughout and these are outlined here for future reference.

## **Amplification and cloning of DNA sequences**

### **PCR amplification of specific DNA sequences**

Target domain and interdomain regions of the sequenced A4 $\nu$ ar gene were amplified for cloning using the polymerase chain reaction (PCR). Forward and reverse primers were designed around domain boundaries previously described, (Smith, Subramanian et al. 2000). Primers were designed to include a restriction site at each end of the resultant product. Each region to be cloned had a blunt-ended 5' site created by using Xmn-1 restriction enzyme or its isochizomer SmaI, and a staggered 3' end to allow directed cloning. Each different PCR reaction was optimised by varying cycle times, annealing temperature and magnesium chloride concentration. Individual conditions are given below. Approximately 100ng of A4 genomic DNA (gDNA) was mixed with PCR buffer (10mM tris pH 8.3, 50mM potassium chloride), 0.3mM each of dATP, dCTP, dGTP, and dTTP, 1.5-3.5 mM magnesium chloride, 1 $\mu$ M of each primer and 1.25 Units of Taq polymerase. Reactions were performed in 50 $\mu$ l volumes.

### **Separation of DNA fragments**

Plasmid DNA extracted from bacterial cells or DNA fragments amplified from genomic DNA were separated and sized by agarose gel electrophoresis. Gels were

poured using between 1-2.5% weight/volume (w/v) agarose in 0.5 x TBE (1 x TBE – 6g TRIS, 5.5g orthoboric acid and 4ml 0.5M EDTA pH 8.0 made up to 1 litre with deionised water (dH<sub>2</sub>O)). Samples were mixed with equal volumes of loading buffer (30% volume/volume (v/v) glycerol, 10mM EDTA pH 8.0, 0.25% (w/v) xylene cyanol and 0.25% (w/v) bromophenol blue) immediately prior to loading. Gels were run in 0.5x TBE for approximately 1 hour until the first dye front was within 2-3cm of the end. On completion, gels were stained for 20 minutes in 0.5µg/ml ethidium bromide in dH<sub>2</sub>O before ultraviolet (UV) visualisation. Fragment sizes were estimated by comparison to appropriate molecular weight markers run in adjacent lanes.

### **Quantification of DNA**

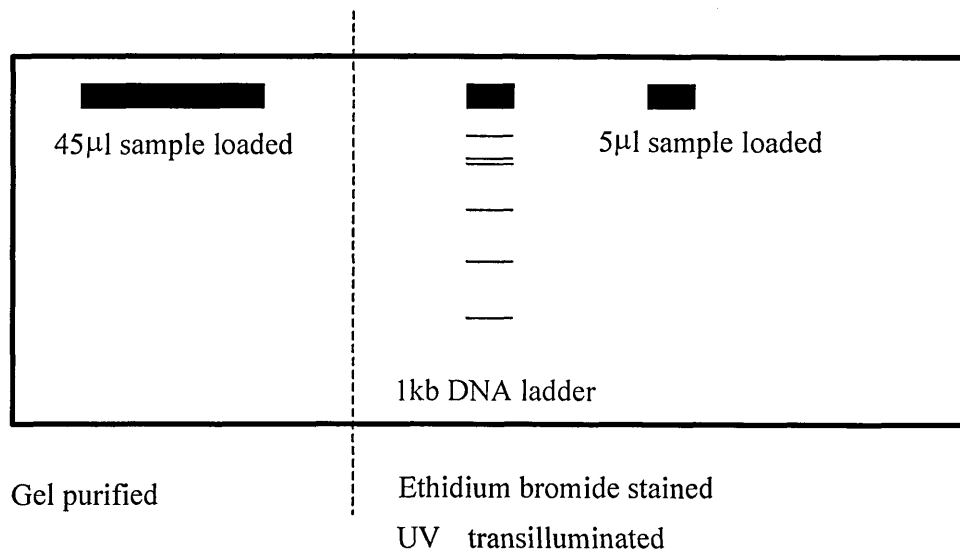
DNA concentration was estimated by agarose gel electrophoresis. Samples were run against a DNA mass ladder consisting of known band sizes, each containing known amounts of DNA. Quantification of DNA in each experimental sample was then estimated by comparison to the appropriately sized band.

### **Post PCR purification of DNA**

To purify the full-length PCR products, a PCR purification kit (Qiagen, Hilden, Germany) was used according to the manufacturer instructions. This technique utilised a silica-gel membrane allowing selective binding of DNA and removal of contaminants.

## **Gel Purification of DNA**

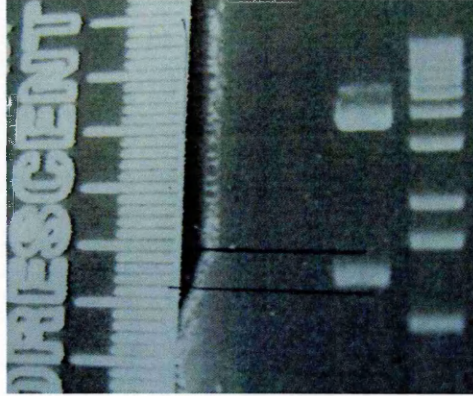
To purify specific DNA fragments from restriction digests, samples were electrophoresed on agarose gel (1% w/v) in TAE buffer, (40mM Tris-Acetate, 1mM EDTA). Because of concerns regarding the effect of UV light exposure and ethidium bromide on the integrity of the DNA purified the following method was used. 50µl samples were split into 5µl and 45µl aliquots and prepared as described previously, samples were mixed with equal volumes of loading buffer (30% (v/v) glycerol, 10mM EDTA pH 8.0, 0.25% (w/v) xylene cyanol and 0.25% (w/v) bromophenol blue) immediately prior to loading. The 5µl aliquots were loaded into a well adjacent to a 1kb DNA mass ladder with the 45µl sample loaded adjacent again (figure 2.4). The gel was run until the dye front was approximately 2-3cm from the end and then cut with a scalpel as shown by the dashed line. The gel containing the DNA ladder and the 5µl sample was stained as before in 0.5 µg/ml ethidium bromide in dH<sub>2</sub>O and then read next to a ruler under UV visualisation (figure 2.5). Using the ruler to measure the distance from the well travelled by the 5µl sample, the unstained 45µl sample, which had not been exposed to UV transillumination, was excised from the gel using a sterile scalpel and transferred to an eppendorf tube. The DNA fragment was then recovered from the agarose gel using QIAquick Gel Extraction Kit (Qiagen, Hilden Germany).



**Figure 2.4**

**Agarose gel showing layout of samples.**

Dashed line indicates where gel was cut.



**Figure 2.5**

**U.V. visualisation of the ethidium bromide-stained DNA.**

The ruler was used to measure the distance travelled by the non-stained DNA and thus inform as to the correct position to excise the DNA from the gel.

## Restriction Digests

Purified vector DNA and PCR products were prepared for ligation by restriction digestion. Vector DNA was additionally treated with phosphatase to prevent self-ligation. Table 2.1 shows the enzymes used and the buffer compositions. When required, double digests using two restriction enzymes followed manufacturer recommendations regarding the choice of a common buffer.

**Table 2.1 Restriction enzymes and buffers used**

Restriction Enzyme	Buffer constituents
BamH 1	50 mM Tris-HCl, pH 8.0, 10 mM MgCl <sub>2</sub> , 100 mM NaCl
Hind III	50 mM Tris-HCl, pH 8.0, 10 mM MgCl <sub>2</sub> , 50 mM NaCl
Pst 1	
Xba 1	
Sma 1	20 mM Tris-HCl, pH 7.4, 5 mM MgCl <sub>2</sub> , 50 mM KCl
Xmn 1	10 mM Tris-HCl, pH 7.9, 10 mM MgCl <sub>2</sub> , 50 mM NaCl

The amount of enzyme used was based on the number of restriction sites per  $\mu\text{g}$  of DNA and the empirically found efficiency of cleavage. Depending on the enzyme, between 0.13 and 1 unit enzyme per  $\mu\text{g}$  DNA when digesting PCR products and between 1 and 5 unit enzyme per  $\mu\text{g}$  DNA when digesting plasmid vector DNA was used at temperatures either 25°C (Sma 1) or 37°C (all other enzymes) for between 4 and 16 hours. For phosphatase treatment 0.03 units of calf intestinal phosphatase (CIP) were added per  $\mu\text{g}$  of DNA for the last hour of restriction digest treatment. DNA was purified by gel electrophoresis and purification as previously described.

## **Ligation**

Plasmid and PCR insert DNA were prepared by restriction digestion with the appropriate restriction enzyme and then gel purified as described. 10ng of vector DNA was incubated with a 1:1 and 1:3 molar ratio of vector:insert DNA. Ligation reaction buffer was 60mM Tris-HCl, pH 7.5, 60mM MgCl<sub>2</sub>, 50mM NaCl, 1mg/ml bovine serum albumin, 70mM β-mercaptoethanol, 1mM ATP, 20mM dithiothreitol, 10mM spermidine and 4 units of T4 DNA ligase. Samples were incubated overnight at 16°C.

## **Bacterial transformation and expression of DNA constructs**

### **Bacteriological Media**

Routine culturing of bacterial cells was performed using Luria-Bertani (LB) medium (10g tryptone, 5g yeast extract and 5g sodium chloride in 1 litre of distilled water, pH 7.0 with sodium hydroxide) and LB-agarose plates (LB medium supplemented with 15g agarose per litre). All media was autoclaved immediately and liquid media was stored at room temperature for up to 1 month, while agarose plates were stored at 4°C. In all experiments utilising the p-Malc2x series of plasmids, ampicillin was added to a final concentration of 100µg/ml from a 50mg/ml stock in dH<sub>2</sub>O. In all experiments using BL21-CodonPlus-Ril competent cells for final expression chloramphenicol was also added to a final concentration of 50 µg/ml from a stock of 50mg/ml in methanol. All cultures were grown in a shaking incubator at 140 rpm and 37°C unless otherwise stated.



### Bacterial Strains Used

There were three strains of *E.coli* bacteria used in these experiments. Strain DH5 $\alpha$  was used for cloning and all transformations and initial expression experiments. Initially strain TB1 was used for final expression of the recombinant protein, however after poor results with expression and purification, BL21-CodonPlus-Ril bacteria cells were used. BL21-CodonPlus-Ril cells contain extra copies of the *argU*, *ileY* and *leuW* tRNA genes in the form of a pACYC plasmid. These genes encode tRNAs that recognise the arginine codons AGA and AGG, the isoleucine codon AUA and the leucine codon CUA, together the tRNAs that most frequently restrict translation of heterologous proteins from organisms with AT-rich genomes. The BL21 expression strains of *E. coli* naturally lack the Lon protease, which can degrade recombinant proteins. They are also engineered to be deficient for a second protease, the OmpT protein. The extra plasmid, pACYC, also has chloramphenicol resistance to maintain the plasmid. The genotypes of these three strains of *E.coli* are outlined in table 2.2.

**Table 2.2 Bacterial genotypes used**

Strain	Genotype
DH5 $\alpha$	SupE44 hsdR17 recA1 gyrA96 endA1 thi-1 relA1 deoR
BL21-CodonPlus-Ril	E.coli B F <sup>-</sup> ompT hsdS(r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) dcm <sup>+</sup> Tet <sup>r</sup> gal endA Hte [argU ileY leuW Cam <sup>r</sup> ]
TB1	F <sup>-</sup> ara $\Delta$ (lac-proAB)[ $\Phi$ 80dlac $\Delta$ (lacZ)M15]rpsL(Str <sup>R</sup> )thi hsdR

### **Transformation of competent bacterial cells**

DH5 $\alpha$  cells were used for initial transformation and replication of recombinant plasmids with TB1 and then BL21-CodonPlus-Ril cells used for final expression. The latter two strains were not used for initial transformations because of poor transformation efficiency. Transformation was performed as follows. 50 $\mu$ l of competent cells were thawed on ice, when using BL21-CodonPlus-Ril cells 2 $\mu$ l of a 1:10 dilution of  $\beta$ -mercaptoethanol was added to the competent cells and mixed gently. This increases the transformation efficiency of these cells. Approximately 20ng of expression plasmid DNA containing the gene of interest was added to each transformation reaction and mixed gently. The reactions were then incubated on ice for 30 minutes. Each transformation reaction was heat pulsed in a 42°C water bath for precisely 20 seconds before being transferred onto ice again for 2 minutes. 500 $\mu$ l of pre-heated LB medium was added to each reaction before incubating them at 37°C for 1 hour while shaking at 225rpm. Using a sterile spreader 100 $\mu$ l of transformed cells were plated onto LB-agarose plates containing ampicillin only when transforming DH5 $\alpha$  cells and ampicillin and chloramphenicol when plating out transformed BL21-CodonPlus-Ril cells. Transformants appeared as colonies following overnight incubation at 37°C.

### **Storage of Transformed Bacterial Cells**

For immediate use, bacteria were grown overnight in LB medium at 37°C before being plated on LB-agarose. These plates were grown overnight at 37°C and then

stored for up to 1 month at 4°C. For longer-term storage, glycerol was added (20% v/v), to overnight cultures and the cultures were then stored at -20°C.

### **Plasmid Isolation and Purification**

Single clones of transformed bacterial were picked from LB-agarose plates and inoculated into 5ml of LB medium containing ampicillin alone or in combination with chloramphenicol depending on the cell type growing. The culture was grown overnight at 37°C. The culture was then harvested by centrifugation for 5 minutes at 10,000 x g in a tabletop centrifuge. The supernatant was aspirated and excess media blotted with a paper towel. 250µl of cell resuspension solution (50mM Tris-HCl, pH 7.5, 10mM EDTA, 100µg/ml RNase A) was added to the pellet and the cells resuspended by vortexing. 250µl of cell lysis solution was added (0.2M NaOH, 1% SDS) and the solution mixed by inversion of the tube. The lysate was incubated at room temperature and observed to clear. In order to inactivate endonucleases released during cell lysis, 10µl of alkaline protease was added and the solution mixed by inversion. A neutralisation solution was added (4.09M guanidine hydrochloride, 0.759M potassium acetate, 2.12M glacial acetic acid) and again the solution inverted. The bacterial lysate was then centrifuged for 10 minutes at 14,000 x g at room temperature. The cleared lysate is then transferred to a silica membrane based minicolumn (Promega, MA, USA) and centrifuged for 1 minute at 14,000 x g, the flow-through is then discarded and the minicolumn reinserted into the collection tube. 750µl of column wash solution (162.8mM potassium acetate, 22.6mM Tris-HCl, pH 7.5, 0.109mM EDTA, 95% Ethanol) was added to the column and centrifugation was repeated. Adding 100µl nuclease-free water to the column and

centrifuging at maximum speed for 1 minute eluted the plasmid DNA. The purified DNA was stored at  $-20^{\circ}\text{C}$ .

## **Sequencing**

Sequencing by automatic sequencer was carried out in order to verify the reading frame and insert boundaries of the DNA constructs produced. Sequencing reactions were prepared using an ABI Prism Dye Terminator Cycle Sequencing kit, following recommended procedures. 500ng of plasmid DNA was mixed with reaction buffer and 3.2pmol primer specific to the insert boundaries spanning the 5' and 3' end of the inserted construct. Reactions were cycle sequenced using a PCR machine. Extension products were purified by ethanol precipitation, with the addition of sodium acetate to 0.3M and 2 volumes ethanol. Tubes were incubated at  $0^{\circ}\text{C}$  for 10 minutes before centrifugation at  $20,000 \times g$  for 15 minutes. Pellets were washed once with 70% (v/v) ethanol before being resuspended in loading buffer and separated on an automated sequencing gel.

Sequence reactions were repeated by the Department of Biochemistry, University of Oxford.

## **Expression of Recombinant Proteins**

### **Maltose-Binding Protein Expression System**

First devised in 1988, (di Guan, Li et al. 1988; Maina, Riggs et al. 1988), the pMAL-2 vector system, (figure 4.1), provides a novel method for expressing and purifying a protein derived from a cloned gene or open reading frame. The cloned gene is

inserted downstream from the *malE* gene of *E.coli*, which encodes maltose-binding protein (MBP), producing a recombinant protein fused to MBP. This vector system utilises the *tac* promoter and the *malE* translation initiation signals to give high-level expression of the fusion protein,(Duplay, Bedouelle et al. 1984; Amann and Brosius 1985) as well as allowing simple purification through the affinity of MBP for maltose,(Kellermann and Ferenci 1982). The vector carries the *lacI<sup>q</sup>* gene, which codes for the lac repressor. This keeps expression low in the absence of isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) induction. The inclusion of an ampicillin resistance gene allowed for selection of transformed bacteria.

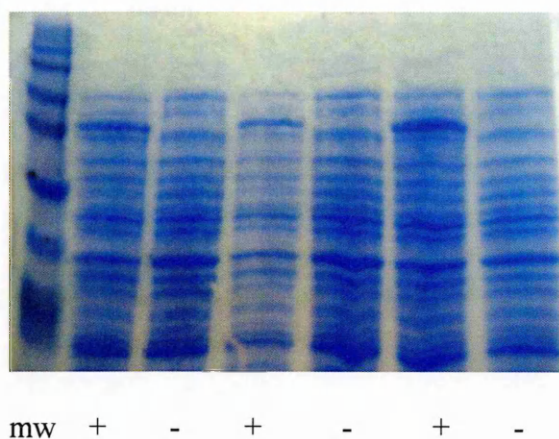
### **Expression of recombinant proteins**

The basic protocol for these experiments was as follows. The bacterial cell cultures consisting of the transformed bacteria were grown to logarithmic phase by shaking at 37°C. IPTG was then added to induce transcription of the recombinant DNA and subsequent translation of the protein of interest. Sufficient time was allowed for the proteins to reach maximal intracellular levels and cells were then harvested, lysed and the recombinant protein of interest purified through use of a maltose column.

Initially small-scale experiments were performed to allow screening of transformed bacterial cells for expression. Once this was established larger scale experiments were performed with the recombinant protein harvested in large quantities. Maltose immobilised on amylose resin was used to purify the recombinant protein.

To screen for expression, LB medium, supplemented with ampicillin and chloramphenicol, was inoculated with transformed bacterial clones and the bacteria

were grown to stationary phase overnight. Following 1:100 dilution with LB medium, bacterial growth was monitored by periodic measurement of the absorbance at 600nm. When the OD<sub>600</sub> was between 0.4 and 0.6, IPTG was added to 0.1mM, after collecting a sample of uninduced bacteria for comparison, and the cultures were incubated for a further 3 hours. 1ml of each culture was then pelleted in a microfuge and 100µl of loading buffer added (2% (w/v) SDS, 0.1M Tris 10% (v/v) glycerol, 10% (v/v) β-mercaptoethanol). The pellets were then boiled for 5 minutes and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) assessed expression (figure 2.6). Conditions for optimum expression were established for each construct and are outlined in chapter 4.



**Figure 2.6**

Expression of three individual clones containing construct CIDR1α. Uninduced (-) and induced (+) lanes are shown.

Once expression had been optimised larger scale purification was commenced. Overnight cultures were seeded with bacterial clones, grown overnight and then diluted 1:100 with fresh LB medium supplemented with 0.2% (w/v) glucose, ampicillin and chloramphenicol and grown in a shaking incubator at varying temperatures until the OD<sub>600</sub> was between 0.3 and 0.6. IPTG to 0.1mM was added and the cultures incubated for the optimal period of time as established. The cells were then pelleted at 6000 x g for 10 minutes. Cell pellets were resuspended in 50ml lysis buffer per 1litre of culture (2ml 1M Tris-HCl, pH 8.0, 10ml 5M NaCl, 0.5ml Tween 20, 0.2ml 0.5M EDTA, 100µg/ml lysozyme, 0.1% Na azide, 1µg PMSF to a volume of 100ml with dH<sub>2</sub>O) and stirred on ice for 1 hour. Sonication was performed using a 10mm diameter probe with a total sonication exposure time of 6 minutes. The lysate was then centrifuged at 16,000 x g for 30 minutes at 4°C. The supernatant was collected and run down a pre-poured amylose resin. Amylose resin is an affinity matrix used for the isolation of fusion proteins with maltose-binding protein. It is poured into a 2.5 x 10 cm column and as it comes pre-swollen in 20% ethanol it is washed with 5 x column volumes of column buffer (10mM Tris-HCl, pH 7.4, 0.2M NaCl, 10mM β-mercaptoethanol, 1mM EDTA). The resin is reported to bind 3mg/ml protein bed volume and so a 5ml column was sufficient for a yield of around 15mg fusion protein / litre of culture. The crude extract supernatant was poured into the column to affect a flow rate of around 1ml/minute. After the extract had ran through, the column was washed with 10 column volumes of column buffer with the fusion protein then eluted by addition of 100mM maltose to the column buffer. The purified protein was collected in 10 x 1ml fractions and each fraction was analysed for presence of the fusion protein by SDS-PAGE. Those fractions

containing the purified fusion protein were aliquoted and snap frozen before being stored at  $-80^{\circ}\text{C}$ .

### Separation and Analysis of Proteins

Protein mixtures were routinely separated on the basis of size by SDS-PAGE, using a modified Laemmli approach. (Laemmli 1970). Acrylamide gels were poured which, when set, allowed migration of proteins differentially, according to size, when a constant voltage was applied. Gels were run under reducing, denaturing conditions to ensure all polypeptides were linearised and secondary structure removed. Bio-Rad mini-protean II cells were used for all experiments.

Solution A	Solution B	Solution C
48ml HCL	48ml HCl	30% (w/v) Acrylamide / 0.8% (w/v) Bisacrylamide
36.3g Tris	5.98g Tris	
0.23ml TEMED	0.48ml TEMED	
dH <sub>2</sub> O to 100ml	dH <sub>2</sub> O to 100ml	

**Table 2.3**

**Composition of buffers used to cast polyacrylamide gels for SDS-PAGE**



	5% Running Gel	10% Running Gel	Stacking Gel
<b>Solution A</b>	2.5ml	2.5ml	—
<b>Solution B</b>	—	—	0.95ml
<b>Solution C</b>	1.7ml	3.3ml	1.25ml
<b>10% (w/v) SDS</b>	0.1ml	0.1ml	0.075ml
<b>10% (w/v) APS</b>	0.1ml	0.1ml	0.075ml
<b>ddH<sub>2</sub>O</b>	5.7ml	4.0ml	5.0ml

**Table 2.4**

**Composition of gels for SDS-PAGE**

Running gels were layered under 50% (v/v) isopropanol until polymerisation was complete. Isopropanol was then removed and the stacking gel was overlaid followed by insertion of the sample comb. Protein samples were mixed with equal volumes loading buffer (2% (w/v) SDS, 0.1M Tris, 10% (v/v) glycerol, 10% (v/v)  $\beta$ -mercaptoethanol) and boiled for 5 minutes before loading. Electrophoresis cells were then filled with running buffer (3g Tris, 14.4g glycine, 10mls 10% SDS, ddH<sub>2</sub>O to 1 litre) and the gels were run for approximately 1 hour at 200V.

Gels were fixed and stained for 30 minutes in stain solution (20% (v/v) methanol, 10% (v/v) acetic acid, 0.05% (v/v) coomassie blue). De-staining was performed in the same buffer without the dye.

## **Quantification of Protein**

Estimates of concentration were obtained by two methods: electrophoresis and a soluble phase protein assay.

For crude estimates of concentration, samples of protein purified as above were compared on an SDS-Page gel to known amounts of bovine serum albumin (BSA). The relative intensity of the coomassie stained bands gave an approximate value for the protein concentration of the sample.

For more accurate quantification, a soluble phase protein assay was carried out using a Protein Assay kit (Bio-Rad). Coomassie Blue was used to stain proteins in aqueous solution with results being read by spectrophotometry at 595nm. BSA was used as a standard. Briefly, protein samples were diluted in PBS and proprietary dye reagent added at a 1:5 dilution. Standard dilutions of BSA were prepared in parallel in PBS. Samples were incubated for 20 minutes before absorbance was read and sample concentrations were calculated from standard curves.

## **Enzyme Linked Immunosorbant Assay (ELISA)**

### **Introduction**

Solid phase assays, in which one reactant has been immobilised to a solid support, such as a polystyrene or polypropylene 96 well plate, are highly versatile. They allow both the qualitative and quantitative determination of antigen or antibody, and are the basis for a wide range of accurate and sensitive measurement tools (Engvall, Jonsson et al. 1971; Engvall and Perlman 1971). There are certain requirements of

the solid-phase system necessary for optimal binding to occur. These include; preservation as much as possible, of the native structure and biological activity of the immobilised antigen, stability of bonding to the plastic surface to enable as little as possible loss of the protein coated and inertness of the solid support such that little non-specific binding occurs.

There are many materials available for the solid-phase support in an ELISA. The most widely used are plastics, most commonly polystyrene. The protein-surface adsorption process is complex with the major driving force behind the interface being hydrophobic interaction. Careful consideration of the buffer used to immobilise the antigen on the plastic is important. Protein stability is influenced by alterations in pH, which can lead to charge neutralisation resulting in denaturation and decreased solubility. Popular coating buffers include 50mM bicarbonate, pH 9.6, PBS, pH 7.2, and Tris-saline pH 8.5. Coating buffers usually do not contain detergent, which can compete with the protein for hydrophobic binding sites on the plastic surface.

Once the antigen has been immobilised it is necessary to reduce as much as possible any non-specific binding. The sensitivity of the assay can be greatly compromised if non-specific binding is substantial. Sources of non-specific binding can include impurities in the protein immobilised, proteins and lipids in the serum sample or contamination during the assay. Secondary antibody can also stick to exposed surface areas on the microwell plate. Minimising non-specific binding can be done in a number of ways. One such way is using a blocking agent such as a solution of

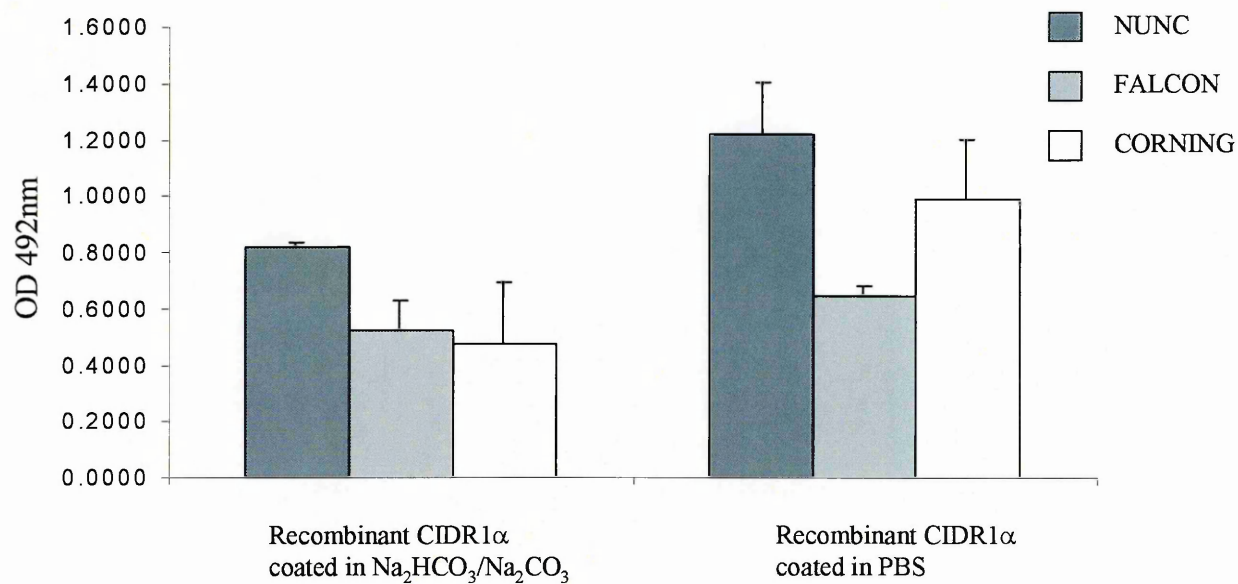
BSA, gelatin or milk. It is worth noting that in some cases, in addition to masking non-specific sites on the plate, blocking agents can also contribute to new sites. For example, albumin is known to adsorb basic immunoglobulins. Previous studies have examined various blocking agents for inhibition of non-specific binding. Vogt *et al* looked at the adsorption of gelatin, non-fat dry bovine milk, casein and BSA onto the surface of polystyrene 96 well plates and scored each in terms of their inhibition of non-specific binding. This was measured by the binding of an antibody-peroxidase conjugate to the surface of the plate. It was found all proteins were able to block at high concentrations but whereas with non-fat bovine milk and casein, blocking was maintained throughout a 10,000-fold dilution, BSA and gelatin exhibited a rapid drop off (Vogt, Phillips et al. 1987).

Another important consideration is the use of detergents. Detergents serve two purposes; they can remove non-specifically bound molecules from the surface of the plate by disruption of hydrophobic bonds formed between the two and they can bind to the surface itself and serve as a blocking agent. For these reasons detergent is included in wash buffers.

### **Selection of plate and coating buffer**

In order to optimise coating of the protein to the plate, the effect of binding to different 96-well micro titre plates from differing manufacturers and coating in two alternative coating buffers at different pH was assessed. The recombinant fusion protein CIDR1 $\alpha$ -MBP was coated at a concentration of 400ng/well in both bicarbonate buffer, (15mM Na<sub>2</sub>CO<sub>3</sub>, 35mM NaHCO<sub>3</sub>, pH 9.3) and phosphate-

buffered saline (PBS), pH 7.2. Each was assessed using three different types of 96-well plate: U-bottom tissue culture grade plates, (Falcon), flat-bottom tissue culture grade plates, (Corning) and flat bottom polystyrene plates, (Nunc). The primary antibody used was mouse monoclonal anti-MBP IgG2a (Dako Ltd.), at a dilution of 1:10,000 with detection being horseradish peroxidase (HRP)-conjugated anti mouse IgG2a at a dilution of 1:5000. Each combination of plate and coating buffer was performed in triplicate and the average OD, read at 492nm, taken after controlling for background binding using PBS or bicarbonate buffer only. Results are shown in figure 2.7. Significantly increased OD values were obtained using PBS as a coating buffer for both the Nunc plates and the Corning plates ( $p=0.0487$  and  $p<0.0001$ , using a paired t-test, respectively). Considering only those wells coated with recombinant protein in PBS, the Nunc plates gave rise to significantly greater OD values than either the Corning or the Falcon plates ( $p=0.0362$  and  $p=0.0167$  respectively). The same analysis was performed with each recombinant protein in turn and consistently coating in PBS using Nunc flat-bottomed 96-well plates give the highest OD values after controlling for background non-specific binding.



**Figure 2.7**

**Graph showing reactivity using different solid-phase supports, coating with 2 different buffers.**

Each condition was done in triplicate and the average taken. Shown are mean values with upper 95% confidence limits. Plates were coated overnight at 4°C and ELISA done according to the method described. The legend at the right-hand side refers to the different types of plate used.

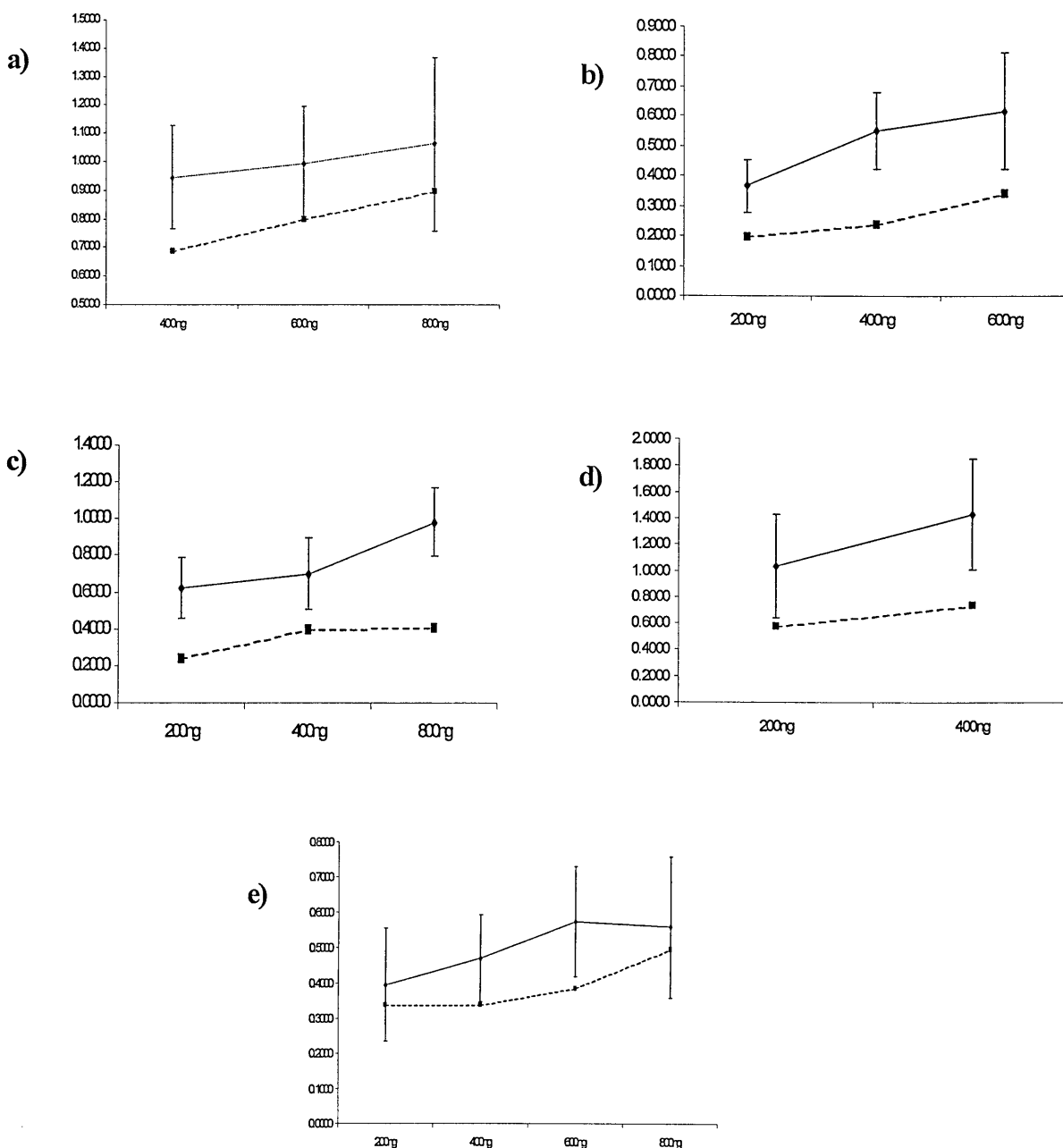
### **Concentration of antigen to coat**

The assay is ideally designed if an antigen, in the correct conformation to optimally bind antibody, takes up the maximum numbers of binding sites on the solid phase. However, as the concentration of antigen in the coating buffer increases, so does the rate of desorption of antigen from the matrix, during the assay. The steric conformation of the antigen may also be altered at high concentrations, thus reducing antibody binding. The optimal concentration of antigen in order to achieve an excess of antigen compared to antibody was determined through the use of a checkerboard titration of diluted protein against a range of positive and negative reference sera. Figure 2.8 outlines the results obtained. Essentially, ELISA's were performed against a range of coating concentrations of each recombinant protein (expressed as ng/well). Reactivity was assessed using a panel of high reacting sera obtained from adults resident within Kilifi District and a panel of sera from non-malaria exposed donors. All sera were measured at a concentration of 1:100. All responses were assessed in triplicate and the average taken. Results are expressed as the mean reactivity for the panel of high reacting serum and the mean plus 3 standard deviations for the non-exposed serum donors. The selection of coating concentration to use for each protein was based on achieving a clear separation in reactivity between the high reacting sera and the non-malaria exposed donor sera and limitations on the amount of protein available. In summary, concentrations used were as follows: DBL1 $\alpha$  - 400ng/well, CIDR1 $\alpha$  - 400ng/well, DBL2 $\beta$  - 200ng/well, DBL4 $\gamma$  - 200ng/well and DBL5 $\beta$  - 400ng/well, (figure 2.8).

## **ELISA Protocol**

An optimal coating concentration, experimentally defined, of each recombinant protein was coated onto individual wells on Nunc transparent, flat-bottomed 96 well plates (Nunc technology) in 100µl PBS. An equivalent molar concentration of MBP alone was coated in exactly the same way as a control. The plates were incubated overnight at 4°C, washed in PBS with 0.05% Tween 20 and blocked with 200µl blocking buffer (10% skimmed milk in PBS with 0.5% Tween 20) for 1 hour at 37°C. The plates were washed again as before and 100µl of human sera (diluted 1:100 with blocking buffer) was incubated in duplicate for 1 hour at 37°C. The wells were again washed and 100µl of HRP-conjugated rabbit anti-human IgG (Dako Ltd.), at a dilution of 1:5000 was added and the plates incubated again for 1 hour at 37°C. Detection was by the addition of O- phenylenediamine/H<sub>2</sub>O<sub>2</sub> (Sigma) for 15 minutes in darkness. The mean absorbance, taken at 492nm, was calculated for each sample after correction for binding to MBP alone. A serum was scored as positive if the corrected absorbance was higher than the mean + 3 standard deviations of 20 negative control sera from UK residents who had never been exposed to malaria.





**Figure 2.8**

### Optimisation of recombinant protein coating concentration

Along the x-axis of each graph are the different coating concentrations of purified recombinant protein. Concentrations are expressed as ng/well on a 96-well plate. Along the y-axis is the absorbance measured at 492nm. The solid lines refer to the average absorbance obtained from 5 high reacting sera from Kilifi District, along with the standard deviations. The dashed lines refer to the average value plus 3 standard deviations of 5 non-malaria exposed donors. All sera were used at a concentration of 1:100. a) DBL1 $\alpha$ , b) CIDR1 $\alpha$ , c) DBL2 $\beta$ , d) DBL4 $\gamma$ , e) DBL5 $\beta$ .

## **Affinity purification of *Pf*EMP1 domain-specific antibodies**

1ml of purified recombinant protein at a concentration of between 0.5 and 1.5mg/ml was dialysed overnight at 4°C against 0.1M NaHCO<sub>3</sub>, 0.5M NaCl pH 8.3, with the medium changed twice using slide-a-lyser dialysis cassettes (Pierce). Using 1g of CNBr Sepharose beads (Amersham Biosciences) for every 5ml of purified protein, the beads were washed three times in 200ml 1mM HCl by gentle centrifugation for less than 2 seconds at 1000g and then removal of supernatant. The dialysed protein and the washed sepharose beads were then mixed in an eppendorf tube and rotated for 1 hour at room temperature, before the excess ligand was removed by washing with 5 volumes of 0.1M NaHCO<sub>3</sub>, 0.5M NaCl pH 8.3. 0.1M Tris-HCl pH 8.0 was added to the beads to a total volume of 1.5ml and the mixture allowed to stand for 2 hours at room temperature. The beads were then again washed with 3 cycles of 5 volumes of alternating buffers as follows: 0.1M Acetate, 0.5M NaCl pH 4.0, 0.1M Tris-HCl, 0.5M NaCl pH 8.0 as per the manufacturer instructions. Five volumes of each buffer were added to the beads and the beads gently rotated, each buffer was then removed by gentle centrifugation for 2 seconds and removal of supernatant as before. A slurry of beads to a final concentration of 75% PBS, 25% settled beads was prepared and to this 1ml of hyperimmune sera, diluted 5 times in PBS, was added. The serum and beads were rolled at room temperature for 1 hour. The mix was then spun for 2 seconds at 1000g and the supernatant removed. The beads were washed 5 times with phosphate buffer comprising 10mM phosphate pH 7.2, immunoglobulins were then eluted using 200µl 0.1M Acetate pH 3.0 and the sample was spun immediately (1000g for 2 seconds). The supernatant was removed and the eluted antibody neutralised immediately with 1mM Tris-HCl pH 8.0.

## **Parasite Culture**

Routine culture of *P. falciparum* parasites was performed according to the methods adapted from Trager and Jensen 1976 (Trager and Jensen 1976). All manipulations were conducted in a tissue culture laminar flow hood using sterile techniques. All surfaces were cleaned regularly with 70% methylated spirit and contaminated disposables rinsed with 1% Virkon.<sup>TM</sup>

## **Culture Media**

Routine culture of parasites was carried out in RPMI-1640 supplemented with 37.5mM HEPES, 20mM glucose, 100µM hypoxanthine, 2mM glutamine, 25µg/ml gentamicin sulphate. The pH was adjusted to 7.2 by addition of sodium hydroxide to pH 7.2 and 10% (v/v) pooled human serum was added (called RPMI-S). For all washes medium without serum was used (denoted RPMI). Pre-sterilised RPMI-1640 medium (Gibco, UK) was used while supplements and other solutions used during the manipulation of cultures, were sterilised either by autoclaving or by passage through a 0.2µm filter and stored at 4°C. Serum from individual donors was tested to ensure that it sustained parasite growth. Serum from 5-7 donors was then pooled, divided into 50ml aliquots and stored at -20°C until required.

## **Preparation of erythrocytes**

Fresh, human erythrocytes of blood group O were obtained from a local blood donor and washed to remove leucocytes and serum. Whole blood was suspended in RPMI to 40% haematocrit. ½ volume of Lymphoprep was added and the suspension was centrifuged at 3000g for 15 minutes at 4°C. Erythrocytes were pelleted by this procedure, leaving leucocytes and serum in suspension and thus able to be aspirated

off. The remaining erythrocytes were washed twice in 10 volumes of RPMI, centrifuging at 3000g for 5 minutes at 4°C after each wash. The final pellet was resuspended in RPMI to 25% haematocrit, and this suspension was stored at 4°C for up to 1 month.

### **Maintenance of parasites in culture**

Parasitised erythrocytes (pRBC) were maintained in RPMI-S in sterile flasks under a modified gas mixture of 96% N<sub>2</sub>, 3% CO<sub>2</sub> and 1% O<sub>2</sub> and stored in an incubator at 37°C. Cultures were routinely maintained at 1-10% parasitaemia in fresh erythrocytes assuming a fivefold rate of trophozoite reinvasion. Suitable culture flasks were chosen to ensure that the depth of medium did not exceed 1cm.

Medium was changed daily according to the following empirical formula:

$$\text{packed cell volume (ml)} \times \text{parasitaemia (\%)} \times 5 = \text{volume RPMI-S required (ml)}$$

For washes cultured cells were centrifuged at 2000g for 5 minutes to pellet them without damaging the pRBC. Washes were performed in 10 volumes of RPMI. All RPMI and experimental materials were pre-warmed to 37°C before use.

### **Assessment of parasitaemia and growth stage**

The routine assessment of the number and stage of parasitised cells in culture was by thin blood smear. Small samples of cultured cells suspended in culture medium were centrifuged briefly in a microfuge. The supernatant was aspirated to give a haematocrit of 25-50% and a drop of this cell suspension was placed on clean microscope slide. Using another clean slide the cell suspension was smeared across

the surface of the slide and left to dry. The slide was then fixed with methanol, dried and stained with Giemsa (10% (v/v) Giemsa stain, 21mM sodium hydrogen phosphate, 4.4mM potassium dihydrogen phosphate, pH 7.35). Slides were stained for 15 minutes before being washed, dried and examined under a light microscope set at 100x magnification under oil immersion. Parasitaemia was determined by counting the number of parasites seen in at least 500 red blood cells. In addition the maturity of parasites was noted by comparison to standard charts (W.H.O. 1991).

### **Preservation and storage of pRBC**

Long-term storage of parasitised cells was achieved by freezing samples in cryopreservative media as individual stabilates following the methods of Diggs and colleagues (Diggs, Joseph et al. 1975). The desired pRBC were centrifuged at 2000g for 5 minutes and the supernatant aspirated. The pellet was slowly resuspended in glycerolyte (57g glycerol, 1.6g sodium lactate, 30mg potassium chloride and 1.38g sodium dihydrogen phosphate made up to 100ml in dH<sub>2</sub>O and adjusted to pH 6.8 with sodium hydroxide) at a ratio of 3 volumes packed cell volume: 5 volumes glycerolyte. Once the first volume of glycerolyte was added, the suspension was incubated for 5 minutes at room temperature before further addition. On completion, the suspension was frozen at -80°C in 200µl aliquots in cryo tubes before being moved to liquid nitrogen (-196°C) for long-term storage.

### **Thawing frozen stabilates**

Frozen stabilates were rapidly thawed at 37°C and then transferred to a sterile polypropylene centrifuge tube (Falcon<sup>TM</sup>; 50 ml) and 0.2mls of 12% (w/v) sodium chloride solution was added dropwise with agitation over 5 minutes. Following

incubation at room temperature for 5 minutes, 5 volumes of 1.8% NaCl (w/v) were added dropwise with agitation over 5 minutes and the cells were then pelleted by centrifugation at 2000g for 5 minutes. The supernatant was aspirated and 5 volumes of 0.9% (w/v) sodium chloride solution was added dropwise with agitation and the cells were spun as before. Finally the cells were washed with 10mls RPMI, centrifuged at 2000g for 5 minutes, the supernatant was aspirated and cells were re-suspended in RPMI-S and put into culture as described.

### **Trypsin treatment of parasitised erythrocytes**

Parasites were washed twice in 10 volumes of RPMI which had been adjusted to the correct pH by addition of sodium hydroxide solution. All trypsin dilutions were performed at pH 7.2. Cells were re-suspended in 10 volumes RPMI containing trypsin at a concentration of 1mg/ml, 100µg/ml or 10µg/ml and incubated for 5 minutes at room temperature. Control cells were incubated in 10 volumes RPMI containing no protease. Trypsin inhibitor was then added at a concentration of 2mg/ml in an equal volume RPMI and the suspensions incubated for a further 5 minutes at room temperature. Cells were then pelleted by centrifugation at 2000g for 5 minutes and washed 3 times in 10 volumes fresh medium prior to use.

### **Parasites**

In this thesis the term isolate will denote a parasite taken from a patient. Line will refer to an isolate established in culture and clone will denote a culture derived from a single cell of a line.

The line A4 was derived from sequential cloning from the Brazilian line IT 4/25/5 (figure 4.3). Two derivations of A4 were used: A4U and A4 40-cycle. A4U was derived by sequential selection for binding to the monoclonal antibody BC6, which is specific to the expressed A4 *var* gene, resulting in a population of infected erythrocytes expressing predominantly one specific *PfEMP1* variant on their surface, A4 *PfEMP1* (Roberts, Craig et al. 1992; Smith, Chitnis et al. 1995). A4 40-cycle refers to parasites allowed to grow in culture for 40 cycles with no selection. More detail on both these parasite clones is given in chapter 3.

The line 3D7 was obtained by limiting dilution cloning of the isolate NF54, which was derived from a patient who acquired malaria in the airport area in Amsterdam, The Netherlands. It is the genome of this parasite which was sequenced in its entirety and recently published (Gardner, Hall et al. 2002).

The clinical isolate P1 was obtained from a 5yr old child admitted to Kilifi District Hospital with a fever and moderately severe malaria. It was kindly obtained and cultured by Dr Margaret Mackinnon. After sample collection, the sample was centrifuged at 693g for 5 min and the red cell pellet obtained washed twice in RPMI 1640 medium. The parasites were cultured until a parasitaemia of 5% was obtained and cryopreserved when the majority of the parasites were at the ring stage.

## **Measurement of antibody responses against the surface of the infected erythrocyte**

Assessment of the reactivity of human sera to the surface of the infected erythrocyte was performed using indirect immunofluorescence as measured by flow cytometry .

### **Flow cytometry**

Mature trophozoite stage pRBC at between 1 and 5% parasitaemia were thawed, as described, or taken directly from culture. They were washed twice in RPMI and the pellet was resuspended at 1% haematocrit in 0.1% bovine serum albumin/phosphate-buffered saline (0.1%BSA/PBS). 1µl of primary antibody, either human sera or BC6 monoclonal antibody, was pipetted into separate wells of a 96-well flat-bottomed plate (nunc) and 9µl of the infected pRBC cell suspension was added to each well giving a final test serum concentration of 1:10. The mixture was incubated at room temperature for 1 hour, following which the cells were washed three times with 0.1%BSA/PBS, spinning at 1500g between each wash to remove the wash buffer. The cells were then re-suspended in 25µl 0.1%BSA/PBS containing the secondary antibody, either rabbit anti-human IgG if testing human sera or rabbit anti-mouse IgG for reactions involving BC6 monoclonal antibody, at a concentration of 1:50. Again the reaction mixture was incubated for 1 hour at room temperature after which a further three washes were performed as before. Finally 25µl of 0.1%BSA/PBS containing a 1:50 dilution of swine anti-rabbit IgG coupled to FITC and 10µg/ml of ethidium bromide was added to each well. A further incubation at room temperature in darkness, for 1 hour was carried out, after which, following a further series of



washes, at least 1000 pRBC were counted on an EPIC/XL flow cytometer (Coulter-electronics, UK).

BC6 monoclonal antibody was isolated from hybridomas generated from mice immunised repeatedly with a triton X-100-insoluble extract from A4 infected erythrocytes (Smith, Chitnis et al. 1995). It is of isotype IgG1 and immunoprecipitates a high molecular weight protein with the characteristics of *PfEMP1* only from clones of the A4 antigenic type. It was used, in this work, to select for a population of infected erythrocytes expressing one dominant *var* gene on their surface, A4 *var*.

Reactivity against the erythrocyte surface was scored in two ways; as mean fluorescent intensity and as percentage of infected erythrocytes positive for FITC staining using the method of Williams *et al* (Williams and Newbold 2003). In detail, mean fluorescence of parasite-infected erythrocytes was determined using the formula:

**(d-c)-(b-a)**

**a** = the mean fluorescence intensity (MFI) of uninfected erythrocytes following incubation in control plasma or isotype control antibody; **b** = MFI parasitised erythrocytes following incubation in control plasma or isotype control antibody; **c** = MFI uninfected cells incubated in immune plasma or test antibody and **d** = MFI parasitised erythrocytes incubated in immune plasma or test antibody.

The specific percentage of cells (either parasite infected or uninfected) demonstrating surface staining was calculated using the formula:

$$[\text{count UR} / (\text{count UR} + \text{count UL}) \times 100] - [\text{count LR} / (\text{count LR} + \text{count LL}) \times 100]$$

Where UR, UL, LR and LL correspond to the upper right, upper left, lower right and lower left quadrants respectively.

### **Statistical analysis**

Data was entered and stored in Microsoft Excel. FACS data was collected using Weasel Software (WEHI, Melbourne, Australia) and converted into Excel files. Databases were converted into Stata 8 files (State Corp, Ca. USA) and analysed in Stata. Detailed and specific statistical analysis is described in each results chapter.

## **Chapter 3**

### **Naturally-acquired antibody responses to erythrocyte surface-expressed antigens**

#### **Introduction**

There is now a substantial body of evidence suggesting humoral responses to the surface of the infected erythrocyte are an important component of acquired anti-malaria immunity (Marsh, Otoo et al. 1989; Bull, Lowe et al. 1998; Giha, Staalsoe et al. 2000). It is thought that individual responses to the surface of the infected erythrocyte are predominantly variant specific, with immunity gained through acquisition of a repertoire of variant-specific responses over time (Jeffery 1966; Hommel, David et al. 1982; Marsh and Howard 1986; Newbold, Pinches et al. 1992; Bull, Lowe et al. 1998; Giha, Theander et al. 1998). In support of this, children develop antibodies following infection that are specific to the infecting isolate, antibodies which were generally absent at the time of the disease episode (Bull, Lowe et al. 1998). However, it is possible that either the diversity in antigenic targets is not limitless or there exists cross-reactivity amongst antigenic targets. Immune sera from African adults resulted in dramatically reduced parasitaemia when given to children with acute malaria living in distant areas (Cohen, Mc et al. 1961; Cohen, McGregor et al. 1961; Edozien, Gilles et al. 1962). Similarly, immune sera from adults from East and West Africa, South America and South-east Asia agglutinated erythrocytes infected with parasites from distant geographical regions (Aguilar, Albrecht et al. 1992). Taken together, these observations may suggest

either the restriction worldwide in the targets of these responses or be an indication simply that certain antigenic determinants have a broad distribution or contain cross-reactive epitopes.

Most studies investigating antibody responses directed against the surface of the infected erythrocyte have used agglutination to measure these responses (Marsh and Howard 1986; Forsyth, Philip et al. 1989; Southwell, Brown et al. 1989; Newbold, Pinches et al. 1992; Smith, Chitnis et al. 1995; Bull, Lowe et al. 1998). Only recently has flow cytometry been widely adopted as a means of evaluating the anti-infected red cell surface response (Piper, Roberts et al. 1999; Staalsoe, Giha et al. 1999; Williams and Newbold 2003). The precise antigenic targets for responses detected by both methods are currently unknown, although the most likely target is the polymorphic protein family *Pf*EMP1 and while correlation between agglutination scores and antibody binding as measured through flow cytometry or surface immunofluorescence has been shown (Marsh and Howard 1986; Kinyanjui 2001), it is not absolutely certain that both methods are detecting the same response.

As already described, the intra-erythrocytic parasite modifies the surface of the host erythrocyte through insertion of parasite derived proteins into the surface membrane which drastically alter the morphology and behaviour of infected cells. They become highly immunogenic and as the parasite matures they adhere to other cells. There are a number of currently identified parasite derived surface proteins including the variant antigens RIFINS and *Pf*EMP1 and the recently discovered SURFIN as well as the modified host protein Band 3 (Leech, Barnwell et al. 1984; Sherman, Crandall et al. 1992; Kyes, Rowe et al. 1999; Winter, Kawai et al. 2005). The most

likely candidate for infected-erythrocyte surface antibody responses and best characterised is a single family of diverse parasite proteins, *PfEMP1*, the features of which have been comprehensively reviewed in chapter 1.

Identifying the specific host immune responses and the molecular targets on the surface of the infected red cell that explain the variant-specific nature of responses and the possibly more conserved or cross-reactive response is of paramount importance, both in understanding host-parasite interactions and the development of a vaccine. In this chapter, heterologous antibody responses to both wild parasite isolates and laboratory parasite lines, including a laboratory clone selected to express one particular variant of *PfEMP1*, are described for two populations resident under differing transmission characteristics on the coast of Kenya. Relationships between recognition of the isolates with age, asymptomatic parasitaemia and the effect of host genotype, specifically  $\alpha$ -thalassaemia and the sickle cell trait (HbAS) are described. Investigation of the variant specificity of each measured response was performed and an attempt made to abrogate antibody binding to the infected red cell surface through protease treatment.

## Aims

1. To describe antibody responses to a laboratory-adapted isolate highly selected for expression of one dominant *var* gene, in a community-based cohort of asymptomatic individuals.
  - a) To describe acquisition of antibody responses with age.
  - b) To examine the differences in acquisition between two areas of differing transmission characteristics.

- c) To examine what effect the presence of microscopically-detectable parasitaemia has on antibody responses.
2. To compare antibody responses to the same laboratory-adapted clone, although unselected, in the same cohort of individuals.
3. To compare antibody responses to two further isolates, the laboratory-adapted clone 3D7 and a clinical isolate obtained from a symptomatic 5 year old child, P1.
4. To assess the specificity of individual antibody responses.
5. To assess the sensitivity of the measured antibody responses to protease treatment.
6. To examine what effect different host genetic markers of resistance to malaria disease have on antibody responses to a laboratory-adapted isolate and a clinical isolate obtained from a symptomatic child.

## **Methods**

### *Study Population*

After obtaining informed consent, sera from 290 asymptomatic individuals were obtained at a cross-sectional bleed in Chonyi and Ngerenya in October 2000 as part of an on-going study (Mwangi 2003). Written consent for the children's participation was given by their parents or guardians. Baseline characteristics of the individuals tested are given in table 3.1.

### *Cross-sectional survey*

A cross-sectional survey was performed in October 2000, when malaria transmission was low. Blood samples were obtained from children by venepuncture and plasma was separated by centrifugation and stored at -80°C. The children were examined for fever (axillary temperature was taken by use of an electronic thermometer), and

their parasitological status was determined by use of a Giemsa-stained thin malaria smear. Individuals were followed up with weekly active malaria case detection and continuous passive case detection. A case of malaria was defined as:

Individuals aged less than 1 year – fever  $> 37.5^{\circ}\text{C}$  plus any parasitaemia

Individuals aged over 1 year – fever  $> 37.5^{\circ}\text{C}$  plus parasitaemia  $> 2500/\mu\text{l}$  (Mwangi 2003)

In order to assess antibody binding in symptomatically unwell children, a separate cohort of 39 children were recruited at presentation with symptomatic non-severe malaria, and followed weekly thereafter for a further 5 weeks. They were examined for antibody recognition of A4U by flow cytometry at time of presentation and weekly thereafter. The methodology used was as for the asymptomatic community cohort with the same positive controls present. All children were aged between 7 and 115 months and were resident in Kilifi district. Samples were collected as part of a study examining the kinetics of antibody response to the infecting isolate (Kinyanjui, Bull et al. 2003).

Table 3.1

Baseline characteristics of individuals at cross-sectional survey

Location	Age (years)											Sex		Microscopically detectable parasites	
	0-1	2-3	4-5	6-7	8-9	10-11	12-15	16-20	21-30	31-50	51-85	Male	Female	Present	Absent
Chonyi	13	25	15	10	11	9	18	10	8	19	10	58	90	55	93
Ngerenya	14	13	21	10	11	11	15	18	9	12	8	70	72	39	103
Total	27	38	36	20	22	20	33	28	17	31	18	128	162	94	196



### *Parasites*

In order to test heterologous antibody responses, three parasite lines were used; laboratory clones A4 and 3D7 and one clinical isolate denoted P1.

#### *A4*

The isolate A4 was derived from sequential cloning from the Brazilian line IT 4/25/5 (figure 4.3). It was provided as a gift to Professor C. Newbold from Dr R.J. Howard. Two derivations of A4 were used: A4U and A4 40-cycle. A4U was derived by sequential selection for binding to the monoclonal antibody BC6, which is specific to the expressed A4 *var* gene. This results in a population of infected erythrocytes expressing predominantly one specific *PfEMP1* variant on their surface, A4 *PfEMP1* (Smith, Chitnis et al. 1995). The extent of expression of this *var* gene can be measured using flow cytometry with BC6. A4 40-cycle, in contrast, was derived from A4 by being allowed to grow in culture with no selection for 40 intra-erythrocytic cycles. Measuring A4 *PfEMP1* expression using flow cytometry revealed that between 72 and 86% of A4U-infected erythrocytes were displaying this protein on their surface compared to between 8 and 12% of A4 40-cycle-infected erythrocytes. Both these cloned parasite lines were a gift from Mr Bob Pinches.

#### *3D7*

The parasite 3D7 was obtained by limiting dilution cloning of the isolate NF54, which was derived from a patient who acquired malaria in the airport area in Amsterdam, The Netherlands. It is the genome of this parasite which was sequenced in its entirety and recently published (Gardner, Hall et al. 2002). Curiously, this

particular isolate is not thought to express *PfEMP1* on the surface of infected erythrocytes to the same degree as other isolates such as, for example, A4 {Sue Kyes, *personal communication*}. A recent paper by Jensen and colleagues describing antibody responses to 3D7 both unselected and selected by sera from children in malaria-endemic areas revealed low reactivity towards the unselected 3D7 as measured by flow cytometry (Jensen, Magistrado et al. 2004). Furthermore, 3D7 displayed markedly less adhesion to CSA and ICAM-1, even following selection for these receptor-binding phenotypes, than isolates of the IT lineage and a range of clinical isolates although binding to CD36 occurred to a high level in both unselected and CSA-selected 3D7 (Beeson and Brown 2004) and {Beeson J.G. *personal communication*}.

#### *Wild Isolate P1*

P1 was obtained from a 5yr old child admitted to Kilifi District Hospital with a fever. It was kindly obtained and cultured by Dr Margaret Mackinnon. After sample collection, the sample was centrifuged at 693g for 5 min and the red cell pellet obtained washed twice in RPMI 1640 medium. The parasites were cultured until a parasitaemia of 5% was obtained and cryopreserved when the majority of the parasites were at the ring stage. Culturing was done according to the methods outlined in chapter 2.

#### *Assessment of anti-infected erythrocyte antibody levels in plasma using flow cytometry*

The serum obtained during the survey was assayed for antibodies to the surface of erythrocytes infected with each of the isolates described; A4U, A4 40-cycle, 3D7

and P1, using flow cytometry. The flow cytometry was carried out as described in chapter 2. All individuals were assayed in duplicate and experiments involving a single isolate were all performed on the same day. Sera was assayed using 96-well U-bottomed plates (Nunc) and results were standardised relative to reactivity against previously screened high positive control sera, present in duplicate on each plate and 20 sera from non-malaria exposed donors from Oxford U.K. Also present on each plate were two blank wells containing no serum sample, for assessment of background non-specific secondary antibody binding and two wells assaying responses of a known high responding serum sample to standard A4 in order to control for day to day variability. A proxy measure of the levels of anti-erythrocyte surface antibodies was taken as the mean fluorescence intensity (MFI) of infected erythrocytes after correction for background binding, as outlined in chapter 2.

### *Host genotype*

To examine the effects host genotype may have on recognition of the parasites tested, a subgroup of individuals were genotyped with regards their sickle and  $\alpha$ -thalassaemia status. Sickle cell status, whether HbAA (wild-type) or HbAS (sickle cell trait) was evaluated using electrophoresis and the common African 3.7kb  $\alpha$ -globin deletion was identified using PCR on DNA extracted by standard methods (Chong, Boehm et al. 2000). All genotyping was performed as part of an ongoing study investigating the relationship between malaria disease and genetic mechanisms of resistance, (Dr Thomas Williams). Genotyping and electrophoresis were performed by Mr Alex Mwacharia, Mr Sammy Wambua and Ms Sophie Uyoga.

### *Statistical analysis*

All statistical analysis was performed using Stata8™ (StataCorp. Ca. USA). The relationship between parasite status at the time of blood sampling and reactivity to the surface of erythrocytes infected with each isolate was assessed using logistic regression. The outcome in every case was positivity for recognition of each isolate as classified using the cut-off of the mean response from 20 non-exposed donors plus 3 standard deviations. Initially the data were explored for additional factors affecting the likelihood of recognising each isolate. In every case these were identified as age (in categories of 6 months duration), location of each individual (either Chonyi or Ngerenya) and previous exposure to malaria infection (estimated by responses to whole schizont extract). The likelihood of recognition of each isolate was then compared in those parasite positive and parasite negative at cross-sectional survey. When the relationship between host genotype and reactivity was assessed, again using logistic regression, parasite status, either positive or negative as determined by microscopy, was included in the model as a possible confounding variable.

When the relationship between parasitaemia and intensity of response was investigated, a multiple linear regression was performed after including the same confounding variables identified as for logistic regression. The outcome variable, MFI, was log transformed prior to inclusion in the linear regression model to normalise the distribution. When investigating the likelihood of recognising a greater number of isolates, an ordered logistic regression was used. Individuals were scored 0, 1, 2, 3 or 4 according to the number of isolates to which they scored positive, the resultant odds ratio obtained gives the likelihood of being in a higher

recognition group comparing two groups of interest, either parasite status, positive or negative or host genotype. The same confounding variables were included as for the binomial logistic regression model. To investigate the relationship between responses to two isolates, again a multiple linear regression was performed after log transformation. To test if exposure alone could account for the positive correlation between responses to two isolates, age (again as a factor of 6 months duration), location, parasite status and responses to whole schizont extract were included as possible explanatory variables.

Where differences in two continuous variables were assessed, no assumptions of distribution were made and the Wilcoxon rank sum test was used to calculate significance. Where more than two variables were compared the Kruskal Wallis test was used. To assess all correlations the Spearman rank correlation coefficient was used.

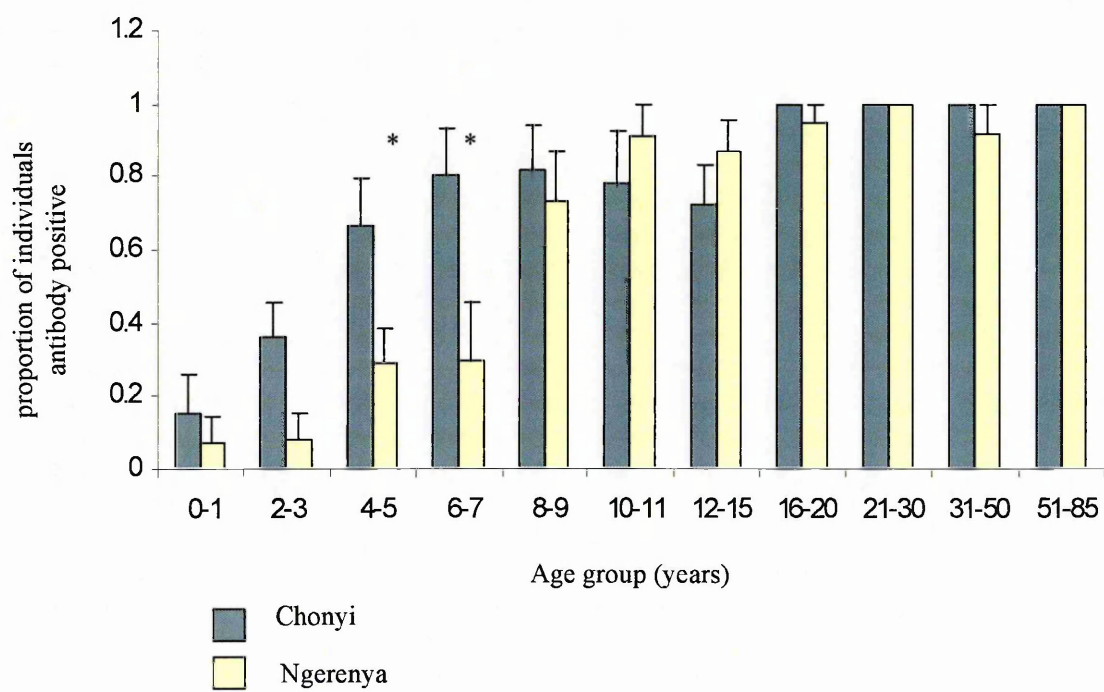
## Results

Sera from 290 individuals resident in both Chonyi and Ngerenya and aged between 6 months and 85 years were tested for their ability to recognise the surface of erythrocytes parasitised with the *P. falciparum* line A4U.

### *Naturally acquired responses to laboratory isolate A4U*

There is marked age-associated acquisition of antibody towards the surface of A4U-infected erythrocytes (figure 3.1). This is the case for those individuals resident within both the high and low transmission study areas (Chonyi and Ngerenya respectively). The chi-squared test for trend across the age groups shown gives a p-

value of  $<0.0001$  for both areas. As will be expanded upon in chapter 6, the risk of suffering an episode of clinical malaria by the definition described reduces significantly at different ages in the two areas. In Chonyi, the risk of experiencing an episode of clinical malaria is significantly less by the age of 5 years, whereas in Ngerenya the risk does not significantly lessen until the age of 10 years. If this test for trend is limited to those age-groups over which the risk of experiencing an attack of malaria becomes significantly less (0.5-5 years in Chonyi and 0.5-10 years in Ngerenya) then there remains a significant age-associated trend of antibody acquisition ( $p=0.0002$  in Ngerenya and  $p=0.0057$  in Chonyi). There is earlier acquisition of antibodies in Chonyi compared to Ngerenya, with responses reaching a plateau of between 80 and 100% of individuals reacting by the age group 6-7 years. This contrasts with the pattern seen in Ngerenya where antibody prevalence continues rising until the age group 10-11 years. It is striking, that in both areas all individuals have antibodies to the surface of erythrocytes infected with this highly selected laboratory clone by late adolescence. If MFI is considered as a marker of the level of antibody binding then there is an increase with age in both areas with individuals in Chonyi recognising the surface of A4U-infected erythrocytes with a greater intensity than individuals in Ngerenya ( $p=0.0044$  Wilcoxon ranksum) (figure 3.2).



**Figure 3.1**

**Proportion of individuals recognising A4U**

Shown are proportion of individuals scoring positive for recognition of A4U as defined in the text within each age category shown. Shown are means and upper 95% confidence intervals.

\*  $p < 0.05$

### *Responses to other isolates*

Those individuals aged 10 years or less were subsequently tested for their recognition of three further parasite isolates: laboratory isolate 3D7, a wild isolate obtained from a symptomatically infected child, P1, and laboratory isolate A4 40-cycle (A4 parasites which have been allowed to grow in culture for 40 cycles with no selection).

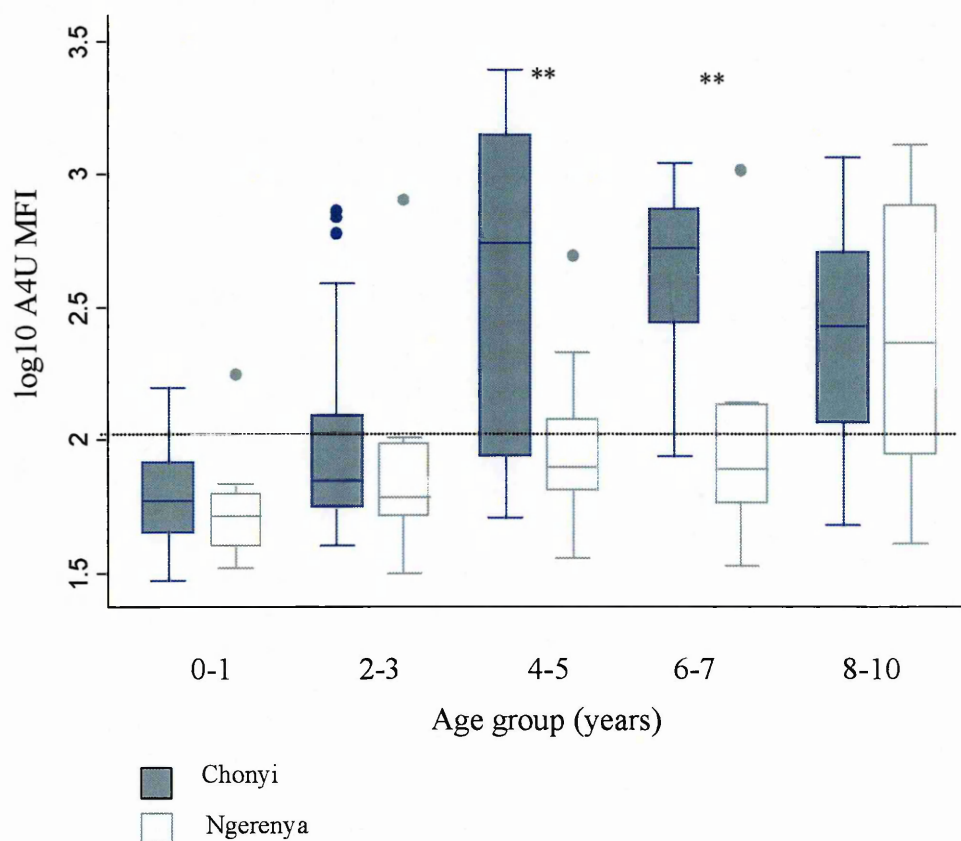
There is evidence of acquisition of antibodies with age to all 4 isolates tested (figure 3.3). The chi-squared test for trend is highly significant for all isolates tested in each area, ( $p < 0.005$  for all isolates, both areas). For all isolates, there is evidence for earlier acquisition of antibodies in Chonyi. Figure 3.4 illustrates the MFI obtained against each isolate, split by location. As is obvious there is a greater intensity of response in the higher transmission area, Chonyi compared with responses from individuals resident in Ngerenya. This is true for all isolates tested. A greater proportion of individuals were positive for recognition of 3D7 overall than any of the other isolates. It was of interest to assess whether this was reflected in greater intensity of responses to 3D7. As is clear from figure 3.4 the median MFI obtained against 3D7 and the wild isolate P1 is lower in most of the age groups tested compared to any of the A4 variants.

Interestingly, the pattern of antibody recognition of A4U was strikingly different amongst symptomatically infected children. Figure 3.5 shows an overview of the results. None of the children showed heterologous recognition of A4U at presentation and only 4 of the children showed any positive response and then only transiently.



*The effect of asymptomatic parasitaemia on recognition of the infected erythrocyte surface*

A previous study from this area, analysing antibody reactivity to variant antigens exposed on the surface of infected erythrocytes, noted that the presence of asymptomatic parasitaemia at the time of serum collection resulted in a higher prevalence of antibodies, as measured by agglutination (Bull, Lowe et al. 2002). A subsequent study using flow cytometry demonstrated a strong association between being parasitaemic and scoring positive for recognition of a range of isolates including unselected A4 (Kinyanjui, Mwangi et al. 2004).

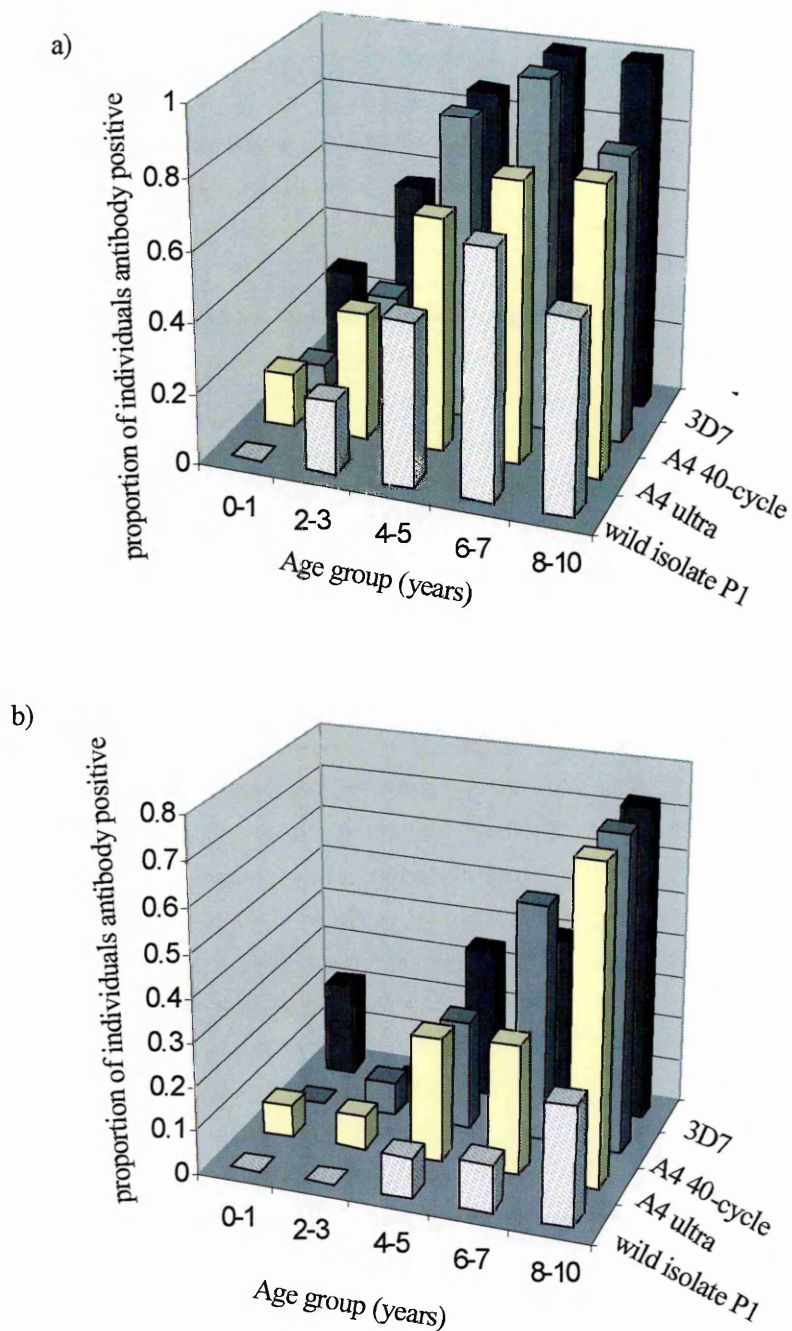


**Figure 3.2**

**Intensity of response to A4U amongst children under 10 years**

Shown are median MFI plus upper and lower 95% confidence intervals after log transformation. The x-axis refers to the age category in years. The dashed horizontal line highlights the cut-off for positivity (the mean of the log transformed MFI from 20 non-exposed donors plus 3 standard deviations).

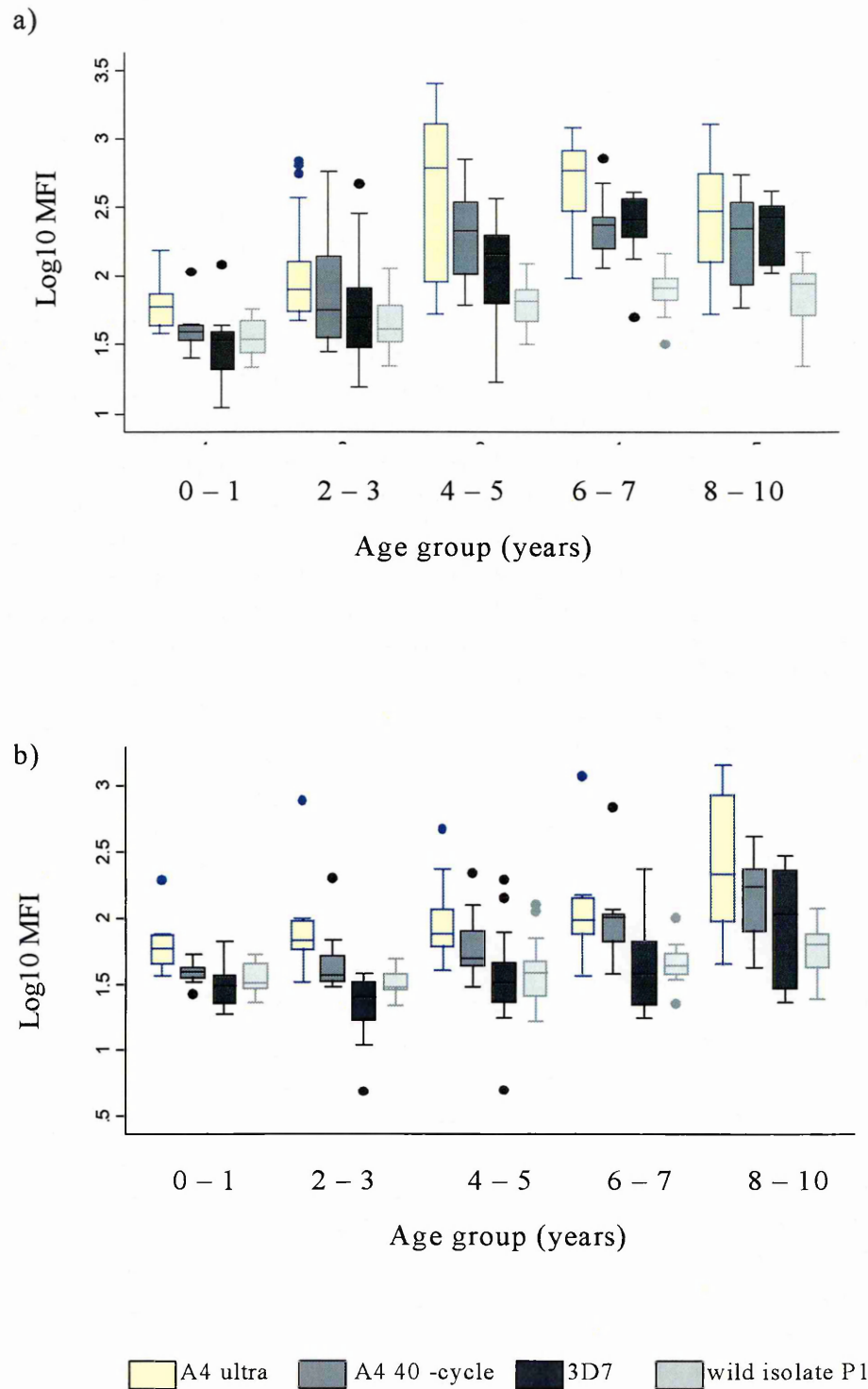
\*\* p<0.005



**Figure 3.3**

### **Responses to all isolates tested**

Shown are the proportion of individuals in each age category shown, scoring positive for each of the isolates tested. Positivity was scored as defined in the text and the absolute value differed for each isolate. a) refers to individuals resident in Chonyi and b) to individuals from Ngerenya.



**Figure 3.4**

**Intensity of responses to all 4 isolates tested**

Shown are median log transformed MFI plus 95% confidence interval of responses to all 4 isolates by age. The key given at the bottom refers to the colours of the boxes.

a) individuals resident in Chonyi and b) individuals from Ngerenya



**Figure 3.5**

**Heterologous antibody responses to A4U at presentation with clinical malaria and thereafter at intervals for 12 weeks.**

The horizontal grey line highlights the cut-off for positivity as defined as MFI greater than the average plus 3 standard deviations of a panel of 20 non-immune donors. Week 1 refers to presentation with acute symptomatic malaria, subsequent weeks refer to weekly follow-up. The numbers above each graph refer to the serial number of each individual child.

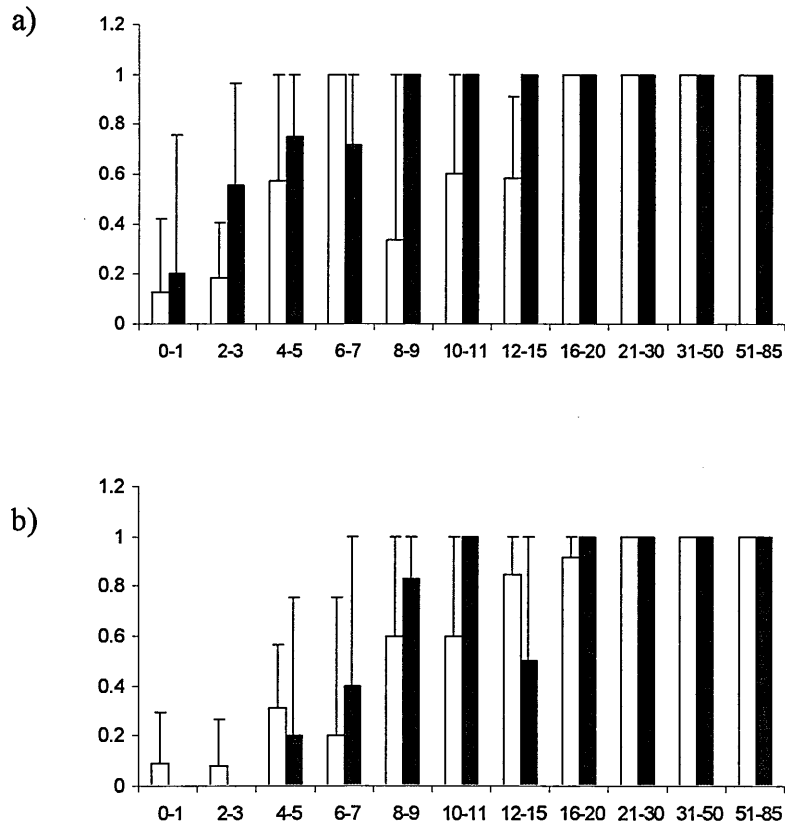
Figure 3.6 illustrates the proportion of individuals in each age group reacting to the surface of A4U infected erythrocytes stratified by parasite status at the time of blood sampling. For those individuals aged less than 10 years, a greater proportion of those parasitaemic score positive (MFI greater than the average MFI plus 3 standard deviations of 20 non-exposed donors from the U.K.) for recognition of A4U. In those over 10 years, there is no difference between the groups due mainly to saturation of positivity. If the effect of parasite status on recognition to each isolate in turn was assessed by logistic regression, adjusting for the effects of age and location on acquisition of antibody responses and exposure as estimated by responses to whole schizont extract, then there is a significant increased likelihood of recognition of all isolates except the wild isolate P1, if an individual was asymptomatically parasitised at the time of cross-sectional survey, (figure 3.7). When MFI was considered as a continuous variable, in a linear regression model adjusting for age and location, (table 3.2), having detectable parasites at cross-sectional bleed resulted in significantly higher MFI against all isolates including P1, the wild isolate.

#### *Correlation of responses between isolates*

In order to assess the specificity of each individual response, scatter diagrams of paired responses, normalised on a log scale, were plotted (figure 3.8). A distinct positive correlation between individual responses to each isolate in turn was observed. This was confirmed by Spearman's rank correlation coefficient (table 3.3). Furthermore, the possibility that these relationships were entirely as a consequence of an individual's exposure to malaria infection were shown to be unlikely by performing a multiple linear regression model examining the relationship between

responses to each isolate controlling for age, categorised as a factor of six month duration, location, parasite status at cross-sectional bleed, location and responses to whole schizont extract as a proxy measure of exposure. From these results it was evident that exposure alone did not explain the very strong positive correlation in responses between the different isolates (table 3.4).

What is also evident from the scatter diagrams is that there are a proportion of individuals showing specificity for one isolate over another. The proportions of individuals in each area scoring positive for antibodies to 0, 1, 2, 3 or 4 isolates are outlined in table 3.5. Also shown are the odds of recognising 0, 1, 2, 3 or 4 isolates according to whether an individual was resident in Chonyi or Ngerenya after accounting for confounders, age, the presence of asymptomatic parasitaemia and exposure (estimated by response to whole schizont extract). It is clear that individuals not showing any antibody response to any isolate were significantly more likely to reside in the low transmission area and conversely those recognising all 4 isolates were more likely to come from the high transmission area, Chonyi.

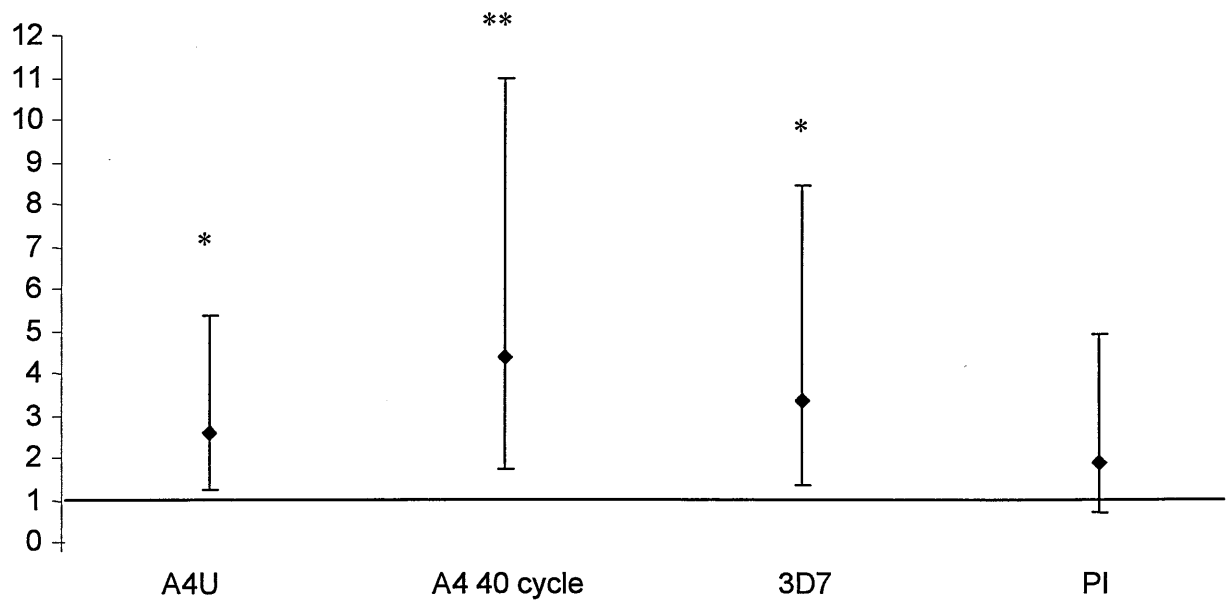


**Figure 3.6**

**Proportion of individuals recognising A4U stratified by presence of parasites**

Each graph illustrates the proportion of individuals in each age category scoring positive for recognition of A4U. Shown are means and upper 95% confidence intervals. The solid grey bars refer to those individuals parasite positive by microscopy at cross-sectional survey and the cream bars to those parasite negative. a) Individuals resident in Chonyi, b) Individuals resident in Ngerenya.





**Figure 3.7**

**Odds ratio of being antibody positive against each isolate tested if parasite positive at cross-sectional survey**

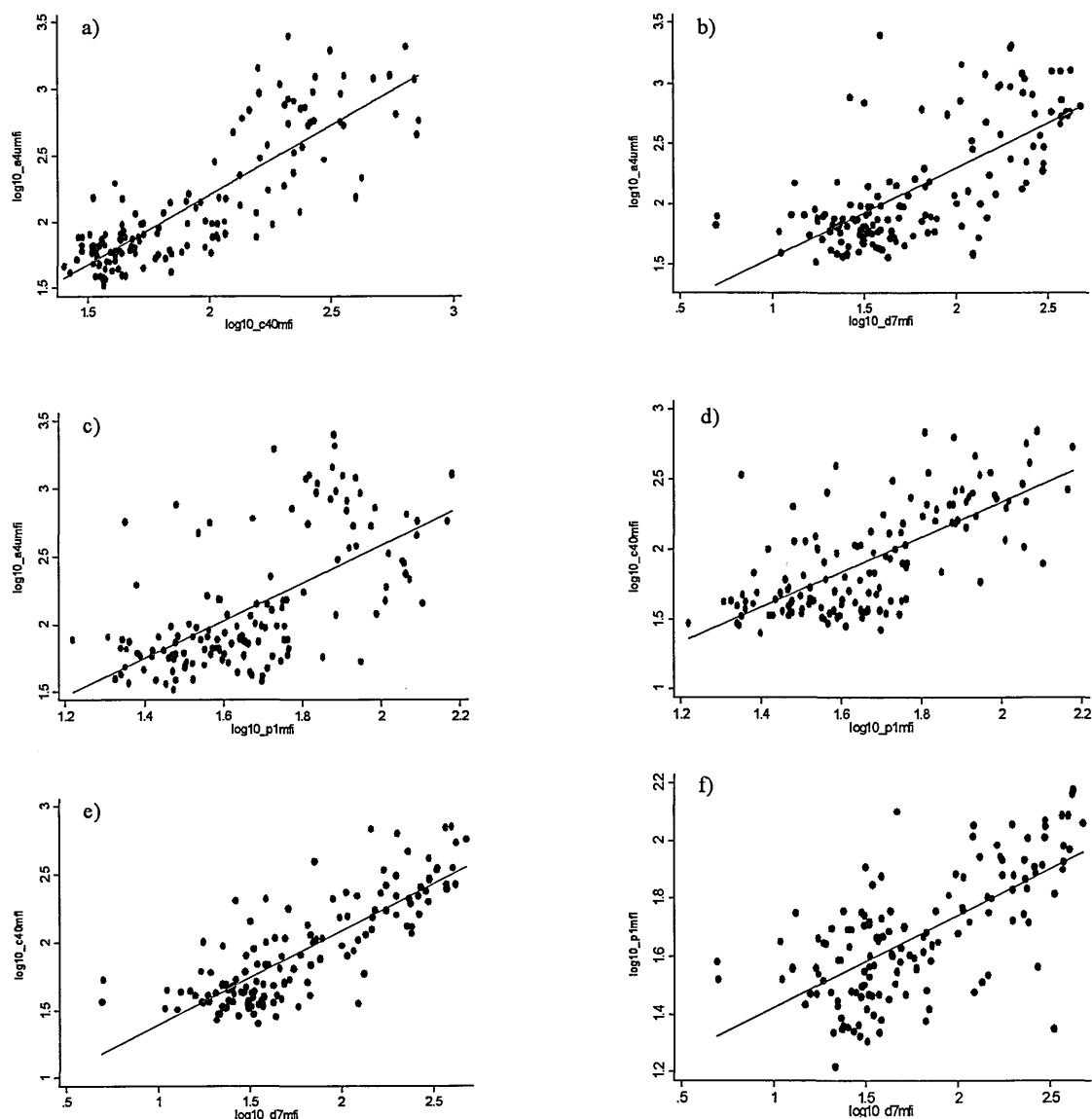
Odds ratio calculated using a logistic regression model controlling for location, age and exposure as estimated by responses to schizont extract. The horizontal line illustrates an odds ratio of one.

\*= $p < 0.05$  \*\*= $p < 0.005$

**Table 3.2**  
**Increase in intensity of response as a result of the presence of parasites**

Isolate	Fold difference in MFI <sup>1</sup>	95% confidence interval	p-value
A4U	1.175	1.02 – 1.36	0.038
A4 40-cycle	1.274	1.16 – 1.40	0.003
3D7	1.198	1.07 – 1.35	<0.001
P1 (clinical isolate)	1.073	1.01 – 1.146	0.031

<sup>1</sup>The results of a multiple linear regression model examining the effect of having parasites on the resultant MFI obtained in response to each isolate in turn. Confounding variables included in the analysis were the age of the individual, the location and an individual’s exposure, as estimated by responses to schizont extract.



**Figure 3.8**

### Inter-isolate correlations

Scatter diagrams showing correlations of individual responses to pairs of isolates. All MFI scores have been log transformed. The best-fit line is shown.

- a) A4U v A4 40-cycle
- b) A4U v 3D7
- c) A4u v P1
- d) A4 40-cycle v p1
- e) A4 40-cycle v 3D7
- f) P1 v 3D7

**Table 3.3****Inter-isolate correlations**

correlation	Spearman's rho <sup>1</sup>	p-value
A4U – A4 40-cycle	0.8408	<0.0001
A4U – 3D7	0.6975	<0.0001
A4U – P1 (clinical isolate)	0.6203	<0.0001
A4 40-cycle – 3D7	0.8163	<0.0001
A4 40-cycle – P1	0.7021	<0.0001
3D7 – P1	0.6711	<0.0001

<sup>1</sup>Spearman's rank correlation coefficients for each pair of comparisons. Shown are the coefficients and the significance values.

**Table 3.4 Relationship between antibody responses to A4U and antibody responses to all other parasite lines**

a) Univariate analysis with no adjustment for confounding variables

Isolate	Coefficient <sup>1</sup>	95% C.I.	p-value
A4 40-cycle	1.05	0.94 - 1.17	<0.001
3D7	0.74	0.62 - 0.87	<0.001
Clinical isolate P1	1.38	1.09 - 1.68	<0.001

b) Multivariate analysis adjusted for age, location, parasite status and exposure

Isolate	Coefficient <sup>2</sup>	95% C.I.	p-value
A4 40-cycle	1.13	0.96 - 1.30	<0.001
3D7	0.71	0.52 - 0.89	<0.001
Clinical isolate P1	0.99	0.64 - 1.33	<0.001

<sup>1</sup>Coefficient calculated using univariate linear regression. The outcome of interest was MFI of antibody responses to A4U after log transformation. In each case the explanatory variable was MFI of antibody responses to each isolate in turn after log transformation.

<sup>2</sup>Coefficient calculated using multivariate linear regression. The outcome of interest was MFI of antibody responses to A4U after log transformation as above. In each case the explanatory variable of interest was MFI of antibody responses to each isolate in turn after log transformation. The coefficient was adjusted for the effect of age (included as a categorical variable of six months duration), parasite status (positive or negative for microscopically detected parasitaemia), location and exposure (as estimated by antibody responses to whole schizont extract after log transformation).

**Table 3.5**

**Specificity of antibody responses**

No of isolates recognised <sup>1</sup>	Number individuals (%) Chonyi	Number of individuals (%) Ngerenya	Odds of residing in Ngerenya compared to Chonyi <sup>2</sup>	95% confidence interval	p-value
0	18 (25)	39 (57.4)	8.10	(2.87 – 22.9)	<b>&lt;0.001</b>
1	10 (13.9)	9 (13.2)	0.73	(0.25 – 2.23)	0.584
2	9 (12.5)	7 (10.3)	0.85	(0.28 – 2.56)	0.777
3	13 (18.1)	8 (11.7)	0.59	(0.19 – 1.77)	0.355
4	22 (30.6)	5 (7.4)	0.16	(0.05 – 0.52)	<b>0.002</b>

<sup>1</sup>Individuals are scored as antibody positive if adjusted MFI is greater than the mean plus 3 standard deviations of 20 non-exposed donors.

<sup>2</sup>Odds ratio is calculated using logistic regression adjusted for age (as a category of six months duration), the presence of detectable parasites and exposure as estimated by response to whole schizont extract. .

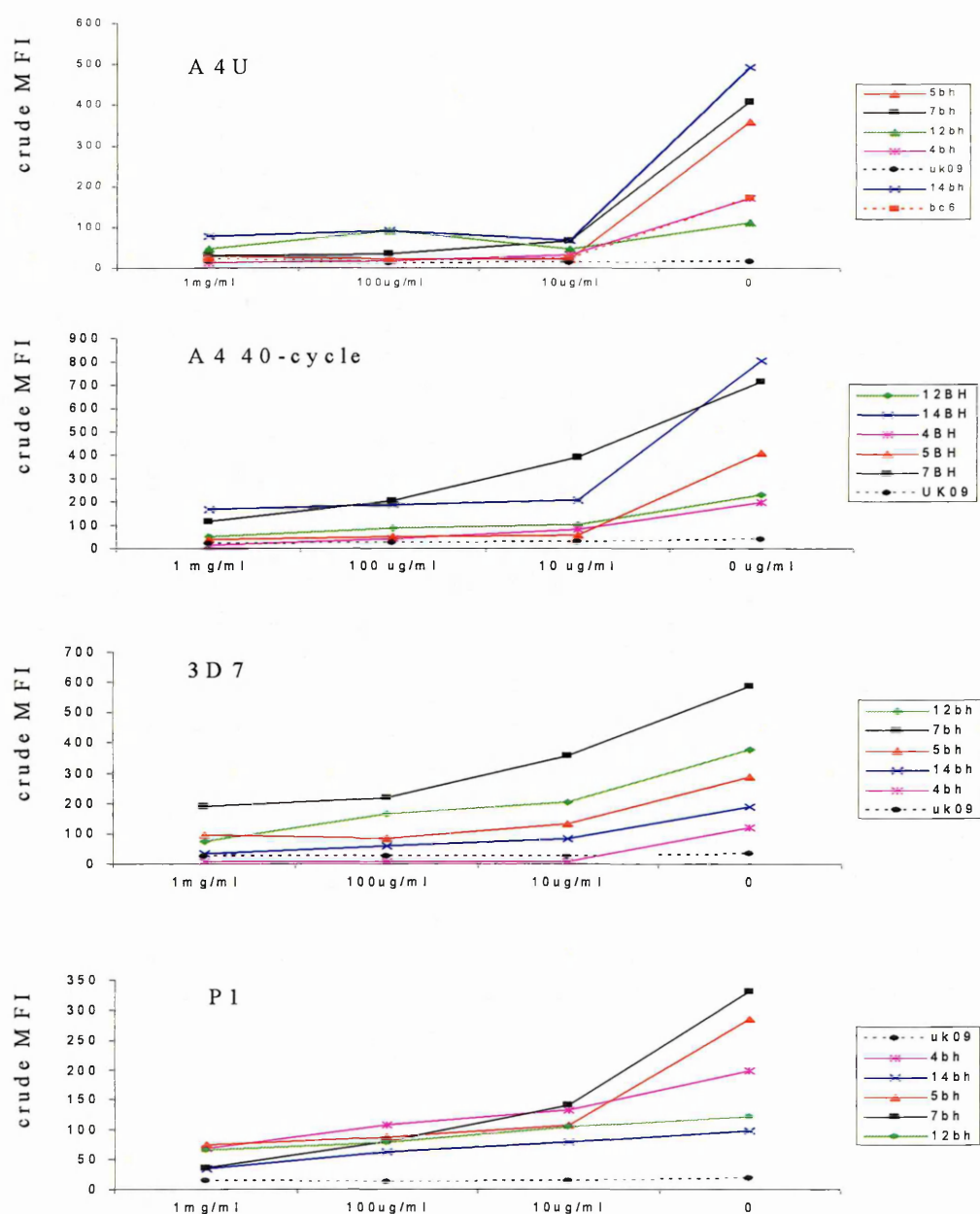
### *Responses to protease-treated infected erythrocytes*

All of the parasite-expressed antigens on the infected erythrocyte surface currently identified are trypsin-sensitive (Sherman, Crandall et al. 1992) (Leech, Barnwell et al. 1984) (Kyes, Rowe et al. 1999) (Winter, Kawai et al. 2005). *PfEMP1* is classically thought to be exquisitely trypsin-sensitive with RIFINs less so (Leech, Barnwell et al. 1984) (Kyes, Rowe et al. 1999). In order to assess whether the antigenic targets binding IgG in this study were also sensitive to trypsin and if so to what extent, each of the four isolates tested were subjected to trypsin treatment. Initially infected erythrocytes were pre-treated with serial dilutions of trypsin with control aliquots of infected erythrocytes treated with RPMI only. All samples were then treated with trypsin inhibitor at the same concentration. Figure 3.9 outlines the results obtained. Whereas A4U appears extremely sensitive to trypsin even at concentrations as low as 10µg/ml, the other 3 isolates tested show less of a marked drop of in reactivity. Responses to all four isolates, however, do show evidence of trypsin sensitivity albeit to different degrees. It was decided to screen all individuals against all isolates pre-treated with trypsin at a concentration of 10µg/ml and repeat this at a concentration of 1mg/ml. Experiments involving individual parasite isolates comparing mock-treated, 10µg/ml and 1mg/ml of trypsin were conducted on the same day. Unfortunately supply of the wild isolate P1 was limited and as such we were unable to test each individual serum against the isolate following trypsinisation.

At 10µg/ml of trypsin, reactivity to both A4U and A4 40-cycle was markedly reduced (figure 3.10). Overall reactivity against A4U was reduced by 84.2% and against A4 40-cycle by 77.1%. As is clear from figure 3.9, the same could not be

said for reactivity against 3D7 with overall reactivity only reduced by 5%. All correlations between isolates were maintained following treatment with 10µg/ml of trypsin, albeit less strongly (table 3.6). Again all correlations remained significant when the effects of exposure were considered in a multiple linear regression as before (data not shown). Testing all individuals against all isolates pre-treated with 1mg/ml abrogated over 93% of the reactivity against both A4 derived isolates and reduced reactivity against 3D7 by 55.6%. Furthermore, no correlations between responses remained significant.

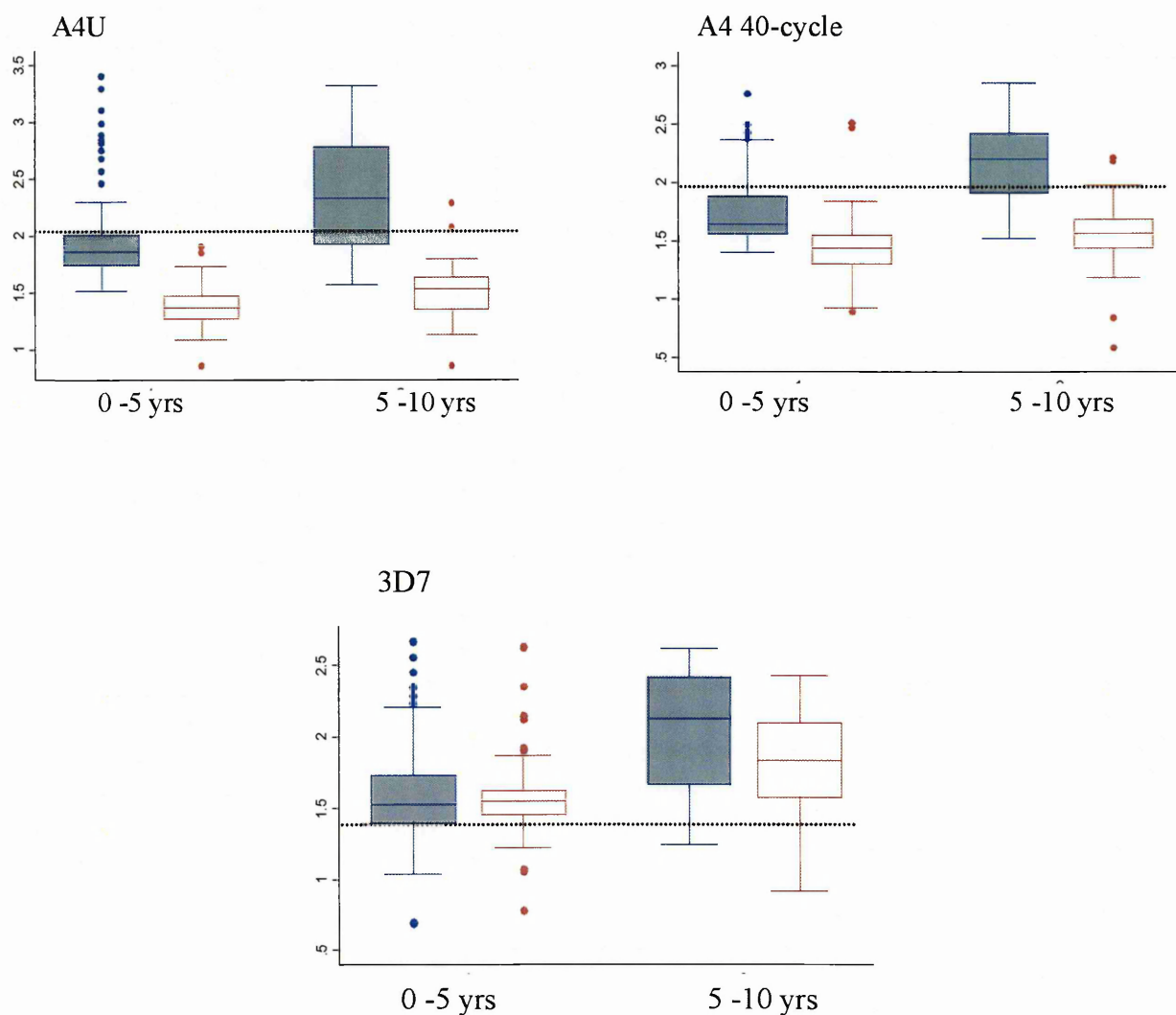




**Figure 3.9**

### **Response to trypsin**

Graphs illustrate the effect pre-treatment of each isolate with serial dilutions of trypsin had on reactivity of a selection of adult sera. The colours are maintained through each graph with the same colour referring to the same serum. The same individuals were tested against each isolate with the exception of A4U where the monoclonal antibody BC6 was also tested. The concentration of trypsin used is plotted along the x-axis with the crude MFI (after correction as detailed in the methods) is plotted along the y-axis.



**Figure 3.10**

### **Response to pre-treatment with 10µg/ml of trypsin**

Box and whisker plots showing median fluorescence intensity following log transformation after 0µg/ml (grey boxes) and 10µg/ml (white boxes) of trypsin. The horizontal dashed lines correspond to the mean response of 20 non-exposed donors against each isolate plus 3 standard deviations, measured against each isolate.

**Table 3.6**

**Inter-isolate correlations following trypsin treatment**

correlation measured	Spearman's rho <sup>1</sup>	p-value
A4U – A4 40-cycle	0.4087	<0.0001
A4U – 3D7	0.4242	<0.0001
A4 40-cycle – 3D7	0.3679	<0.0001

<sup>1</sup>Spearman's rank correlation coefficients for each pair of comparisons between isolates pre-treated with 10µg/ml of trypsin. Shown are the coefficients and the significance values.

### *Host genotype and responses to the infected erythrocyte surface*

The effect of host genotype, both  $\alpha$ -thalassaemia and HbAS, on recognition of A4U, a measure of heterologous response was examined in a subset of individuals.

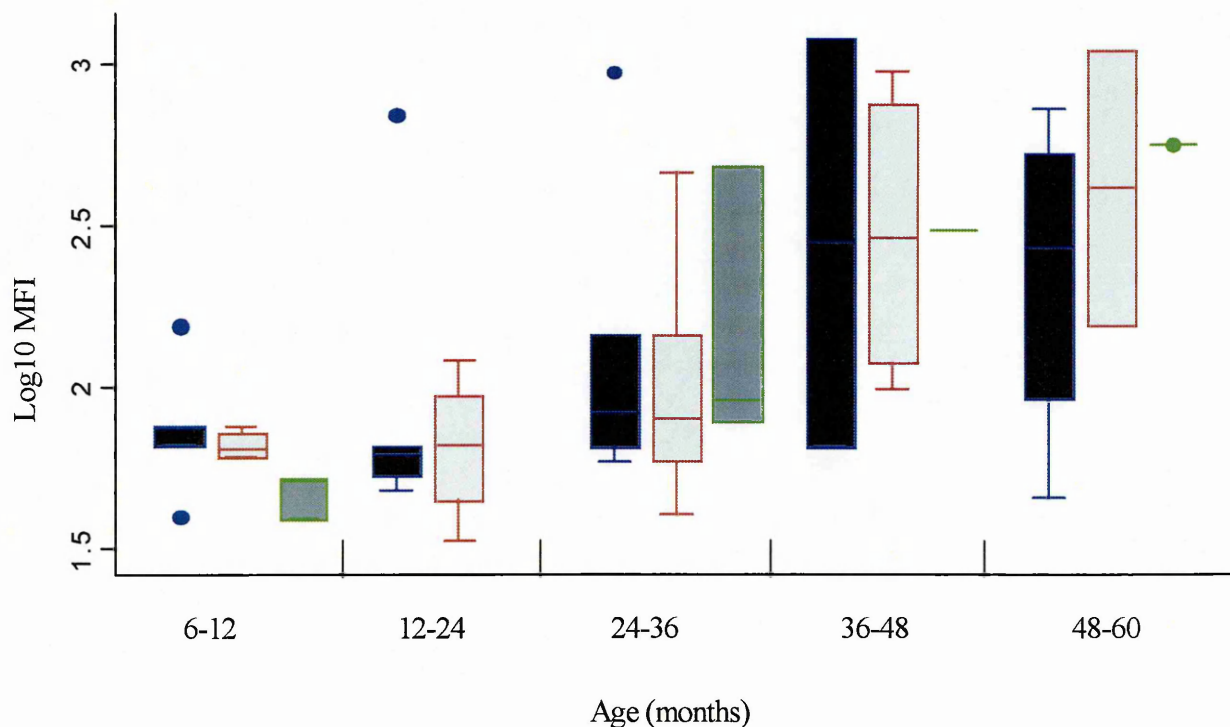
#### *a) $\alpha$ -thalassaemia*

There was no difference in intensity of recognition of the surface of A4U infected erythrocytes between those with normal  $\alpha$ -globin genotype, those heterozygous for  $\alpha$ -thalassaemia or those with homozygous  $\alpha$ -thalassaemia, in any of the age groups tested (figure 3.11). Looking at the median MFI obtained over all the age groups and locations tested; there was no significant difference between any of the three groups (figure 3.12). This was also the case when responses against A4 40-cycle, 3D7 and the wild isolate P1 were examined (figure 3.13). It is important to note that there was no difference in the proportion of individuals parasite positive between the three groups ( $p=0.6686$ , Kruskal Wallis), nor was there any significant difference in the spread of ages represented by the three groups, ( $p=0.2118$ , Kruskal Wallis) (figure 3.14).

If individuals were categorised according to the number of isolates they recognised, 0, 1, 2, 3 or 4 and an ordered logistic regression done with the number of isolates recognised as the outcome, then after controlling for exposure, age, location and parasite status, individuals with either hetero- or homozygous  $\alpha$ -thalassaemia were 56% less likely to be in a higher recognition group, although this result was at the threshold for statistical significance (OR 0.44 (95% CI 0.19 – 1.00)  $p=0.05$ ).

*b) HbAS*

Within the cohort of individuals originally sampled and described thus far, there were only 6, amongst those genotyped, with HbAS. We thus established a new cohort of individuals purely to look in more detail at antibody responses in those with HbAS. 162 people from Ngerenya were sampled in October 2000 as described previously. These individuals ranged in age from 9 months to 75 years and comprised 104 people with genotype HbAA and 58 people with HbAS. There was no significant difference in the proportion of individuals parasite positive ( $p=0.7589$ , Wilcoxon ranksum) although there was borderline significance in the spread of ages represented, ( $p=0.0541$ , Wilcoxon ranksum) between the two groups with individuals in the HbAS group slightly older (mean age of HbAS individuals 139.5 months (95% CI 93.7 - 185.4 compared to mean age of HbAA individuals 96.5 months (95% CI 73.9 - 119.1)) However if we limited the analysis to only those individuals less than 10 years there was no significant difference between the two groups ( $p=0.8963$  Wilcoxon ranksum). Of the 58 individuals with HbAS, 12 had a normal  $\alpha$ -globin genotype, 28 had either heterozygous or homozygous  $\alpha$ -thalassaemia and 18 were not typed.



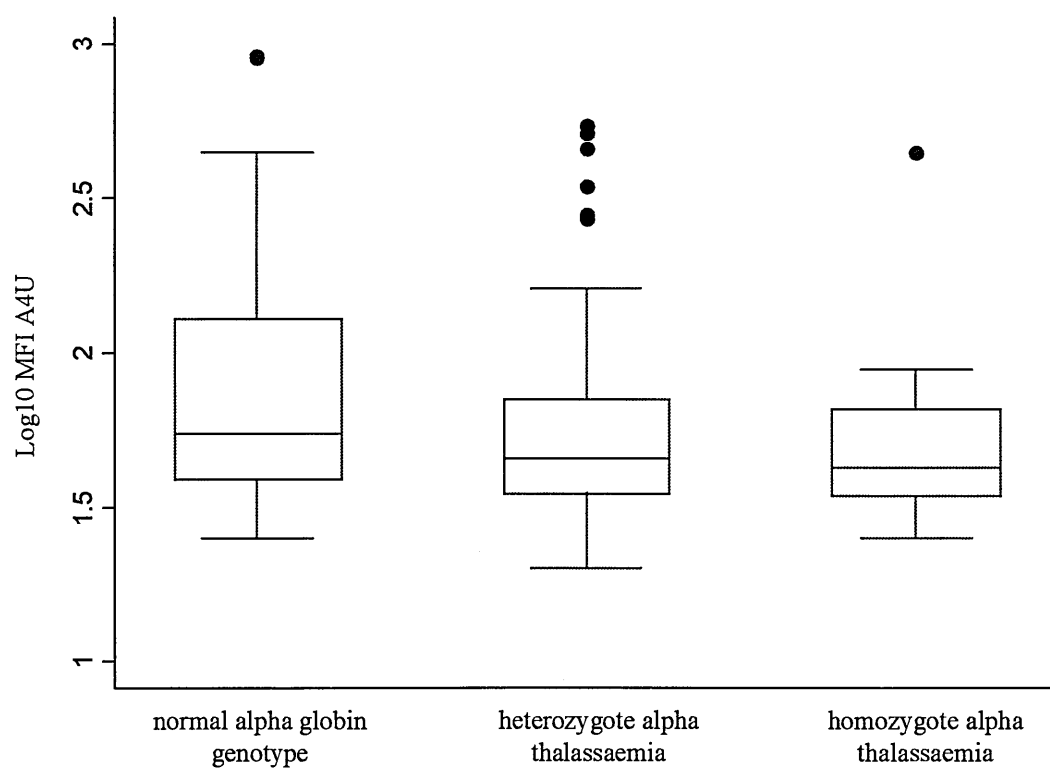
- Homozygous alpha-thalassaemia
- Heterozygous alpha-thalassaemia
- Normal alpha-globin genotype

**Figure 3.11**

### **Intensity of response to A4U by age and thalassaemia genotype**

Box and whisker plot of median responses to A4U stratified by thalassaemia genotype. Responses are ordered by age. Included are individuals from both Chonyi and Ngerenya

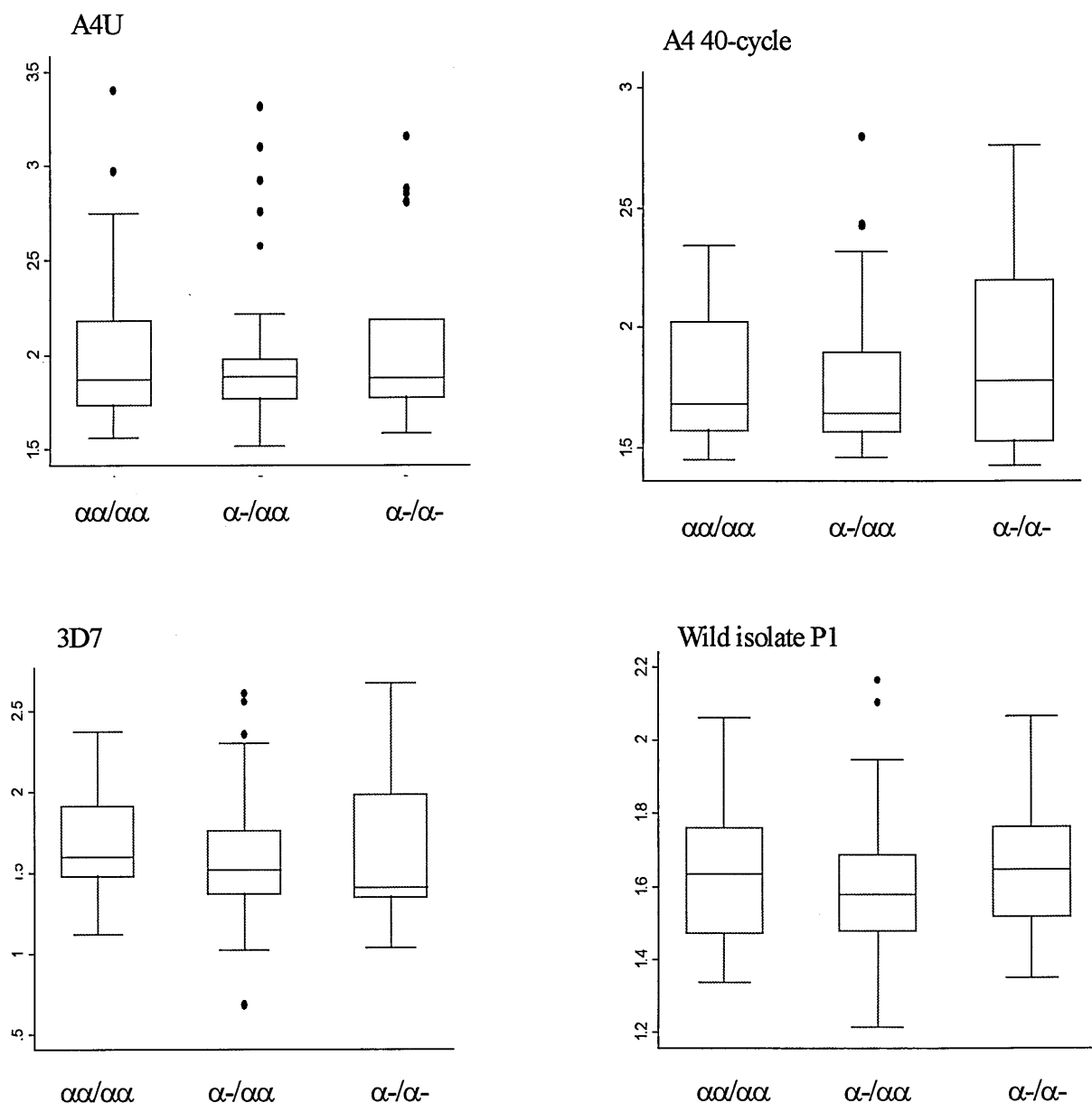
No differences reach significance.



**Figure 3.12**

**Intensity of recognition of A4U by genotype**

Box and whisker plot showing median MFI obtained against A4U by genotype. There were no significant differences between the groups.

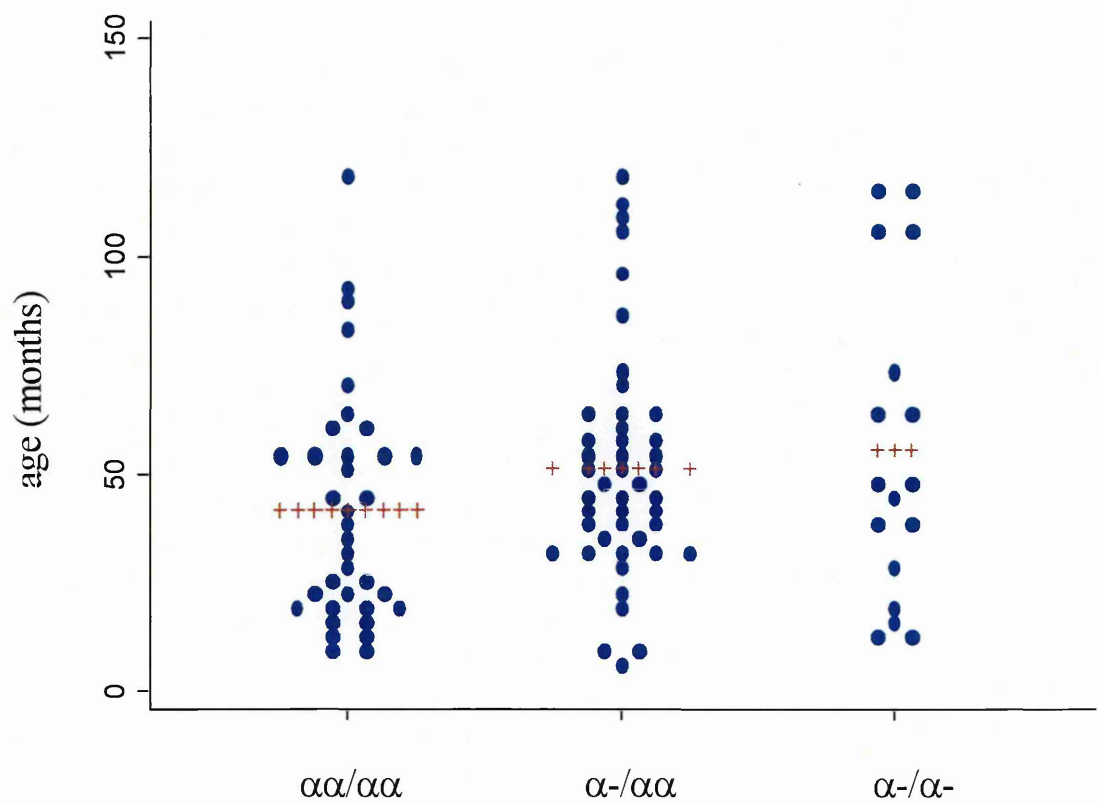


**Figure 3.13**

### **Responses to all four isolates stratified by thalassaemia genotype**

Box and whisker plots showing the median MFI after log transformation and 95 % confidence limits obtained to each isolate stratified by thalassaemia genotype. Included are individuals aged less than 10 years from both areas. There were no significant differences between the groups.





**Figure 3.14**

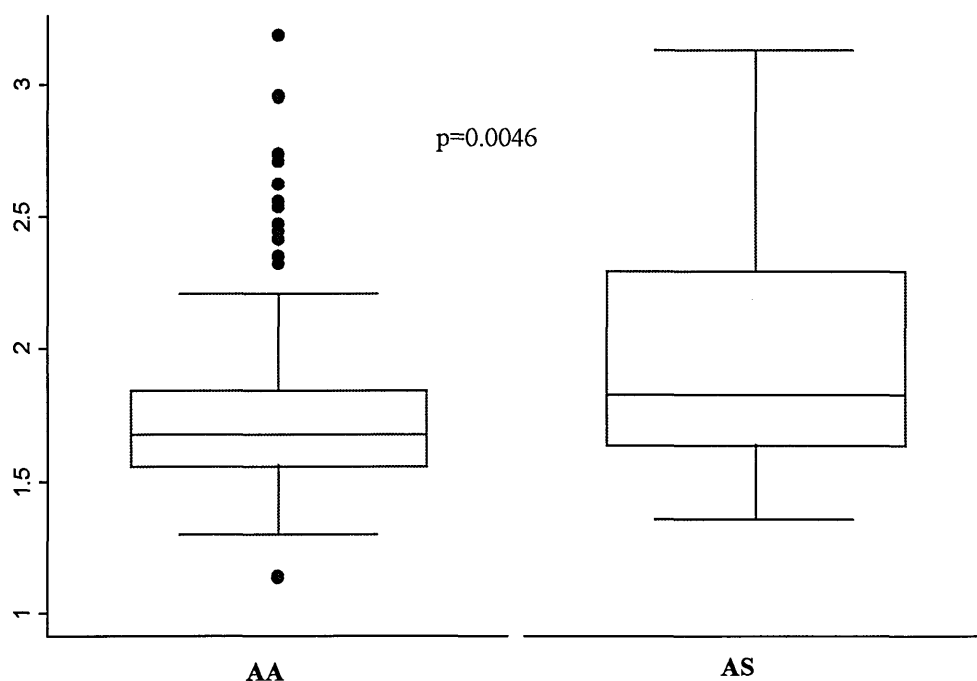
**Ages of all individuals within each thalassaemia group**

Dot plot showing equality of the ages represented within each genotype. The red crosses illustrate the mean. There was no significant difference between the groups.

Considering the population as a whole, those with HbAS genotype had significantly higher MFI for responses against the A4U-infected erythrocyte surface than those with HbAA (figure 3.15). If median MFI is plotted against age, then in those aged 5 years and younger there is a significant increase in response amongst those with HbAS genotype (figure 3.16). This is not the case in the older age groups. If this 0-5 year age range is looked at in more detail, then the maximum difference in response between the two populations, HbAS and HbAA occurs in those aged less than 36months, ( $p=0.0045$ , Wilcoxon ranksum) (figure 3.17). In a multiple logistic regression controlling for the effects of age and parasite status on antibody acquisition as well as exposure, using responses to whole schizont extract as a proxy measure, then the odds ratio of recognising the surface of erythrocytes infected with A4U parasites in HbAS individuals compared to wild-type (HbAA), was 2.47 (95% CI 1.07 – 5.71)  $p=0.034$ .

To assess whether this trend was associated with responses to other isolates, sera from the same individuals were tested for recognition of a clinical isolate, P3, taken from a 5 year old child with moderately severe malaria. Overall there was no significant difference in the level of MFI between HbAA and HbAS individuals although the trend was again higher in the HbAS group (figure 3.18). Assessing response by age revealed no significant patterns of responses across the age groups comparing both genotypes (figure 3.19). The odds of recognising P3 if HbAS genotype compared to HbAA did not reach significance in a multiple logistic regression model, (OR 1.67 (95% CI 0.63 – 4.51)  $p=0.304$ ).

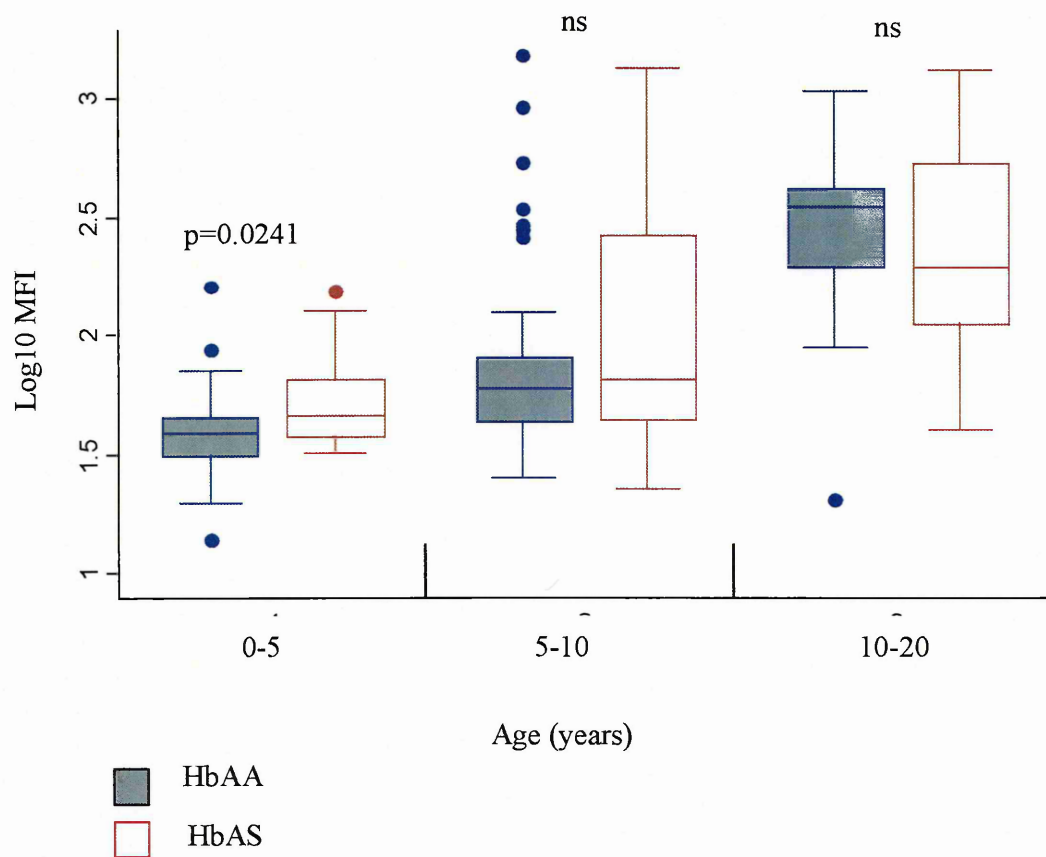
Individuals were then categorised according to whether they recognised 0, 1 or 2 isolates and an ordered logistic regression model was used to assess whether or not those with HbAS genotype were more likely to recognise a greater number of isolates, compared to wild-type HbAA individuals. After including confounding variables; age, parasite status and exposure as measured by responses to whole schizont extract, the odds ratio of being in a higher category, (ie recognising 2 isolates compared to 1 or recognising 1 isolate compared to 0 or recognising 2 isolates compared to 0) if HbAS compared to HbAA was 2.43 (95% CI 1.18 – 5.02)  $p=0.017$ .



**Figure 3.15**

**Intensity of recognition of A4U stratified by sickle cell genotype**

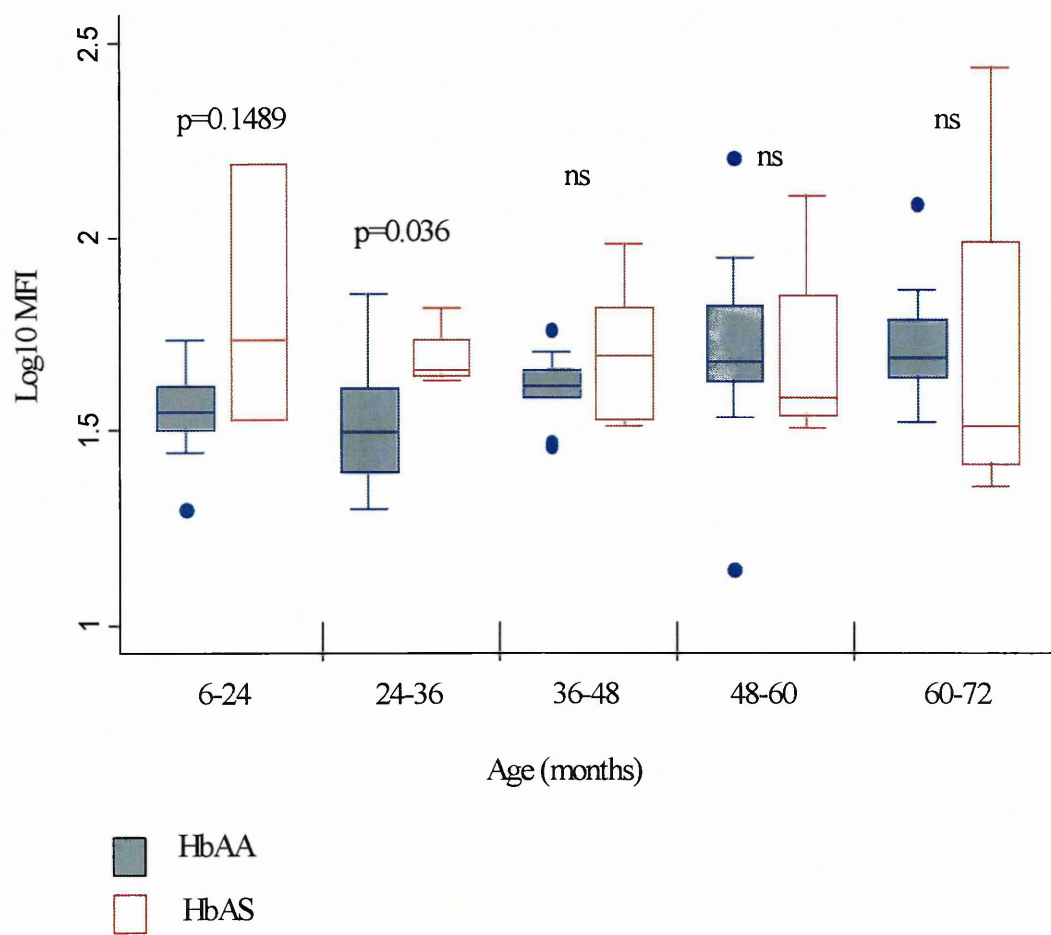
Box and whisker plot showing the median log transformed MFI obtained in response to A4U according to whether individuals were of the genotype HbAA (wild type) or genotype HbAS (sickle cell trait). The p value given refers to the result of a Wilcoxon ranksum analysis comparing the two groups.



**Figure 3.16**

### Intensity of recognition of A4U according to age and genotype

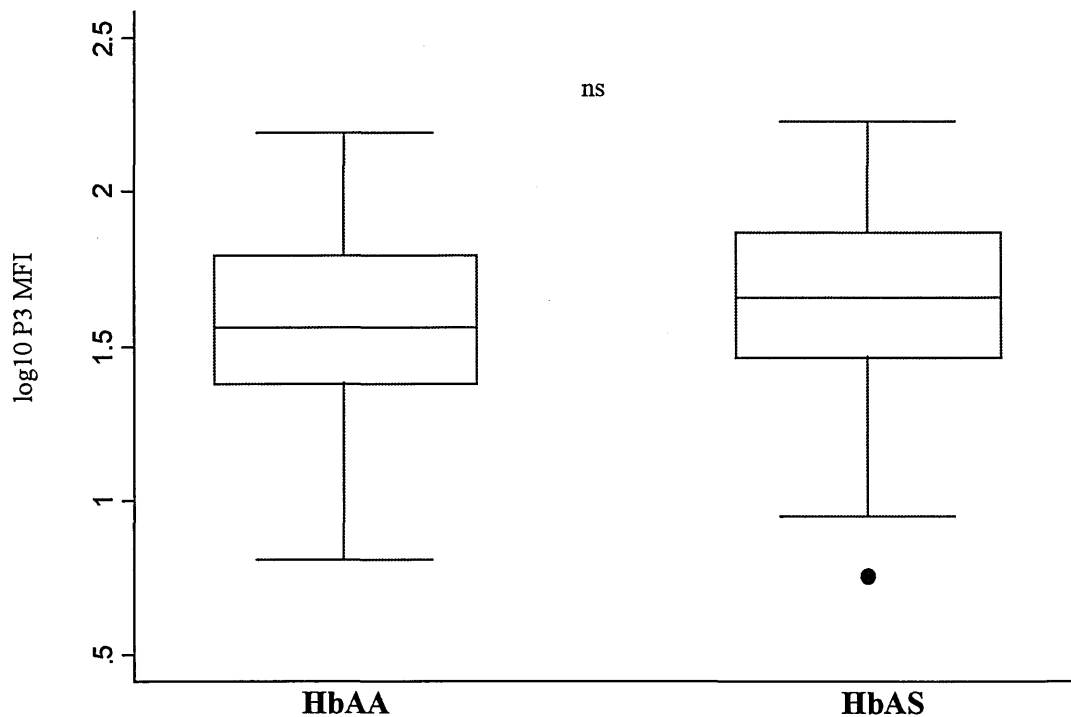
Box and whisker plot showing median MFI according to age and sickle cell genotype. The p values refer to the Wilcoxon ranksum test.



**Figure 3.17**

**Intensity of recognition of A4U in the youngest age groups, according to genotype**

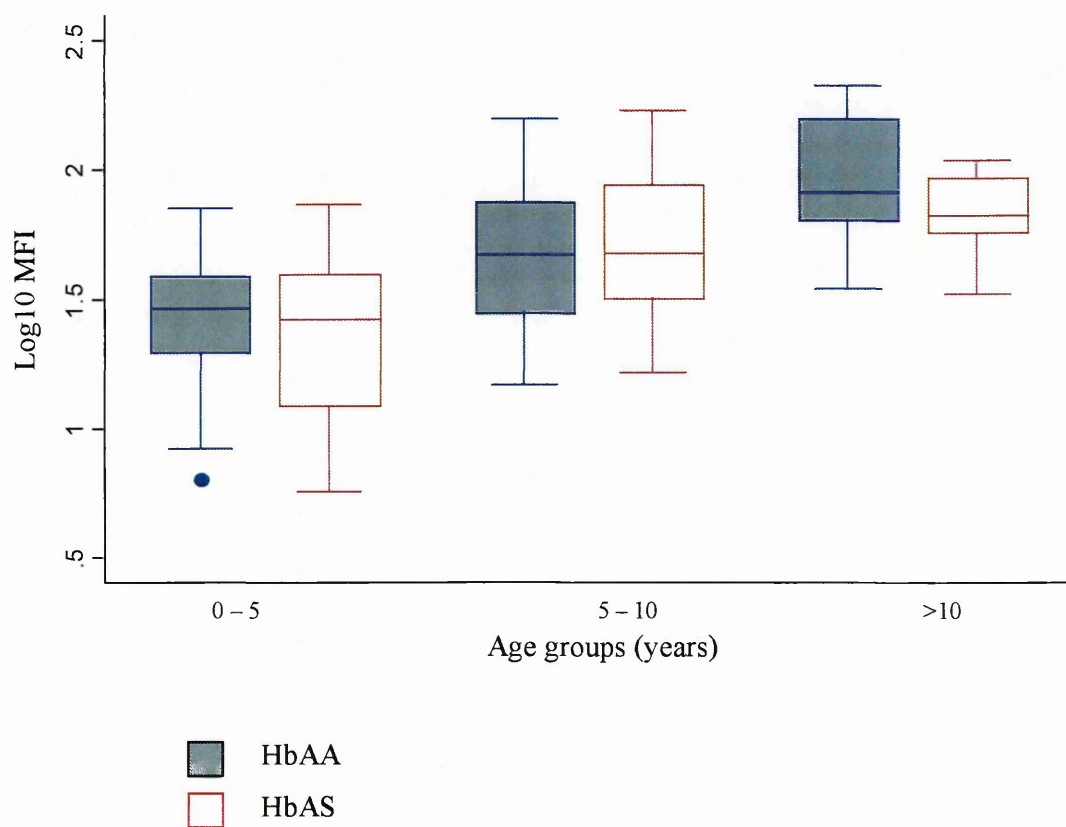
Box and whisker plots of responses according to sickle cell status, in children less than 5 years. P values refer to the Wilcoxon ranksum test.



**Figure 3.18**

**Intensity of recognition of clinical isolate P3, stratified by sickle cell genotype**

Box and whisker plot showing the median log transformed MFI obtained in response to wild isolate P3 according to whether individuals were of the genotype HbAA (wild type) or genotype HbAS (sickle cell trait). The p value given refers to the result of a Wilcoxon ranksum analysis comparing the two groups.



**Figure 3.19**

**Intensity of recognition of clinical isolate, P3, according to age and genotype**

Box and whisker plot showing median MFI according to age and sickle cell genotype. There were no significant differences.



## Discussion

In this chapter individuals were studied at a single point in time for the ability to recognise a panel of isolates including 3 laboratory clones and a clinical isolate. Previous work has described the narrow specificity of agglutinating antibodies induced by clinical infection (Marsh and Howard 1986; Bull, Lowe et al. 1998). Other studies have described induction of antibody responses to heterologous isolates following infection as measured by both agglutination and flow cytometry (Iqbal, Perlmann et al. 1993; Giha, Staalsoe et al. 1999; Giha, Staalsoe et al. 1999; Ofori, Dodoo et al. 2002). The individuals studied in this chapter were all asymptomatic at the time of cross-sectional survey with a proportion in each area parasitaemic. Parasitaemia was identified only by microscopy and as a result there will be a proportion of individuals within the parasite negative group, with parasite loads too low for detection by this method. As all individuals were surveyed at the end of the low transmission season in this area, those parasitaemic will contain some people harbouring chronic infections persistent since the previous high transmission season although as transmission in this area is perennial a number will be newly acquired infections. By using the laboratory isolates A4 and 3D7 the antibody responses measured will be presumed heterologous, that is directed against targets other than those present within the infecting parasite population, in those with detectable parasites. In this way these antibody responses can be thought of as a marker of the individual's breadth of response.

The parasite A4U is a particularly interesting isolate to study responses to. Through selection with the monoclonal antibody BC6, it consists of a population of infected erythrocytes where the majority are expressing on their membrane surface one

dominant *var* gene, A4 *PfEMP1*. The characteristics of this variant of *PfEMP1* are given in chapter 4. This contrasts with A4 40 – cycle which consists of a far more heterogeneous group of expressed *var* genes. When responses to A4U were measured by location and across the ages measured, a striking age-associated acquisition of antibody responses was observed. This was true in both the high and low transmission area. Considering we are potentially measuring responses to a single variant of *PfEMP1*, it is somewhat remarkable that in Chonyi by the age of 6-7 years and in Ngerenya by the age of 10-11 years almost 100% of those tested show evidence of recognition of this isolate (figure 3.1). Furthermore, having an asymptomatic parasitaemia resulted in a greater prevalence of antibodies amongst those parasite positive compared to parasite negative, a pattern persisting until early adolescence (figure 3.6). The association shown here between the presence of parasites and antibodies to this heterologous isolate may reflect an overlap between the antibody repertoire induced by chronic infection and epitopes present within the expressed A4 *var* gene. The very narrow specificity of antibody responses induced following symptomatic clinical infection has been detailed before (Marsh and Howard 1986; Bull, Lowe et al. 1998). It may be that responses generated to isolates other than the infecting isolate target alternative more cross-reactive or conserved epitopes or that the ability to maintain chronic infections is associated with a broader range of antibody specificity due to chronic infections switching through a broader range of variants. An alternative possibility, suggested by Bull and colleagues, remains that a subset of immuno-dominant epitopes exist against which responses are generated early in infection rather than the weeks subsequent to disease (Bull, Lowe et al. 2002), antibodies which could potentially be boosted by chronic infections. Perhaps A4 *PfEMP1* has within it such epitopes. What was striking, was

that when a group of children were examined for their heterologous recognition of A4U, using the same methodology and positive controls, at the time of presentation with clinical non-severe malaria and then weekly thereafter for 5 weeks, only 4 children demonstrated any recognition of this isolate and then only transiently (figure 3.5). This is a result in stark contrast to the prevalence of anti-A4U antibodies described in this asymptomatic community cohort of individuals.

As with responses to A4U, the presence of parasites at the time of cross-sectional survey resulted in a significantly increased likelihood of recognising both A4 40-cycle and 3D7, but not the wild isolate P1 (figure 3.7). Although there was significantly increased intensity of response in the presence of parasites against all 4 isolates (table 3.2).

Previous studies looking at the recognition of unselected A4 amongst children in this area under the age of 10 years demonstrated age-associated acquisition measured by both flow cytometry and agglutination (Kinyanjui 2001). Antibodies were less prevalent than found in this study with a maximum recognition of around 40% by the age group 8-10 years. However differences in methodology and analysis of the flow cytometry acquisition data make any direct comparisons impossible. What was clear from the study described above was that overall, children recognised all field isolates to a greater extent than the laboratory isolates tested, a finding not seen in this work. The field isolate, P1 in this work by contrast was recognised to a lesser degree than the laboratory isolates tested. This is somewhat unexpected as clinical isolates may contain within them many variants and as such we would have expected more prevalent recognition within the community. This particular isolate came from

a symptomatic 5 year old child and previous screening by flow cytometry using a pool of children under 10 years, all parasite negative had revealed a response 81% that of the maximal response generated by screening a pool of adults from this area (data not shown). Thus it may have been expected that the prevalence of recognition be greater amongst the younger aged individuals. If the results are expressed as a percentage of maximal response however, then by the age of 10 years, the median response generated was 79% of maximum.

Previous studies have demonstrated very little reactivity to 3D7 (Jensen, Magistrado et al. 2004){Francis Ndung'u *personal communication*}. It was therefore surprising to note the high overall prevalence of antibodies to this isolate amongst individuals less than 10 years in this cohort (figure 3.3). When intensity of response was estimated by MFI it was clear that IgG binding to this laboratory-adapted isolate was actually substantially lower than responses to either of the A4 derived isolates (figure 3.4). It is not clear why responses to 3D7 should be lower than those found to other isolates. It has been suggested that there is less *PfEMP1* expressed on the surface of 3D7-infected erythrocytes, possibly as a result of a protein transport deficit {Sue Kyes *personal communication*}. Certainly, previous attempts to measure *PfEMP1* yield through Western blotting revealed a lower yield than other isolates {James Beeson *personal communication*}. A recent study demonstrated that parasites of the 3D7 lineage, even those selected for rosetting, had none of the complex vesicles shown in other parasite lines to transport both *PfEMP1* and RIFINs from the parasitophorous vacuolar membrane to the surface of the infected erythrocyte. Instead the parasite 3D7 relied on less complex smaller single vesicles

(Haeggstrom, Kironde et al. 2004). The importance of this to surface expression of both *Pf*EMP1 and RIFINs is as yet unknown.

Recent studies from this area have highlighted the marked protective effect both  $\alpha$ -thalassaemia and the sickle cell trait (HbAS) have on the likelihood of an individual suffering from malaria (Williams, Mwangi et al. 2005; Williams, Mwangi et al. 2005; Williams, Wambua et al. 2005). Within this population, around 15% of individuals have HbAS with the  $\alpha$ -thalassaemia allele frequency at around 0.5. Whereas the protection afforded by the thalassaemia genotypes, both hetero and homozygous is complete and constant from birth, {Thomas Williams *personal communication*}, individuals with HbAS genotype show increased protection with age up until the age of 10 years, a result suggestive of an underlying acquired immune component (Williams, Mwangi et al. 2005). It was hypothesised that this age-associated protection was a result of enhancement of both innate and acquired protective immune mechanisms.

Although the marked protective effect of both sickle cell trait and  $\alpha$ -thalassaemia on the incidence of clinical malaria is well documented in this area (Williams, Mwangi et al. 2005; Williams, Wambua et al. 2005), the precise mechanism of protection by both genotypes is unknown.

It has been hypothesised that increased antibody binding to the infected  $\alpha$ -thalassaemic erythrocyte surface may contribute to the protection afforded by  $\alpha$ -thalassaemia (Luzzi, Merry et al. 1991; Luzzi, Merry et al. 1991; Williams, Weatherall et al. 2002). The increased antibody binding seen was not thought to be

as a result of *Pf*EMP1-specific binding as there was no difference in mAb BC6 binding, specific for A4 *Pf*EMP1 as mentioned, between wild-type and heterozygous  $\alpha$ -thalassaemic red blood cells (Williams, Weatherall et al. 2002). In this study, we found no difference in recognition of each individual isolate between the two groups, either hetero or homozygous  $\alpha$ -thalassaemia, or wild type, however this is perhaps not surprising as this increased antibody binding to the infected red cell may be a function of the effects of the  $\alpha$ -globin deletion on the red cell membrane and in this study all erythrocytes used were wild type.

With sickle cell trait the situation is different. In this study individuals were significantly more likely to recognise A4U-infected erythrocytes, at least in the younger age-groups (figure 3.16). Although no increased recognition of a randomly selected clinical isolate was seen, in an ordered logistic regression individuals were more likely to recognise more isolates if they were HbAS compared to HbAA. These results support earlier work where a significantly higher titre of anti-infected erythrocyte antibodies in children with HbAS compared to HbAA was found (Marsh, Otoo et al. 1989), and more recent work whereby the presence of HbAS genotype was associated with enhanced recognition of two heterologous clinical isolates among Gabonese children (Cabrera, Cot et al. 2005). Interestingly the fact that it is the younger age groups which show increased recognition of A4U-infected erythrocytes is at odds with the increasing protection afforded by the presence of the sickle trait with age in children under ten. Undoubtedly the protection afforded by this genetic trait is a result of a multitude of factors and is not explained by a single phenomenon.

It is interesting to speculate what might be the target for the antibody responses thus described. Previous work has highlighted the variant-specificity of responses measured by flow cytometry directed against the infected red cell surface (Staalsoe, Giha et al. 1999) and that the adhesion phenotype of the infected erythrocyte correlated well with the expressed *var* gene (Smith, Chitnis et al. 1995). Also by using parasites with a deletion on chromosome 9 and therefore an inability to express *PfEMP1* and in all likelihood other antigens, on the surface of the erythrocyte, Piper and colleagues demonstrated marked abrogation of fluorescence intensity, (Piper, Roberts et al. 1999), a finding also seen after trypsin treatment of the infected red cell, (Williams and Newbold 2003). Thus it would appear the most likely candidate for recognition on the infected erythrocyte is *PfEMP1*.

In this study, by comparing individual responses against the isolates described we found strong correlation in responses (figure 3.8). Whilst this may be expected for comparisons made between A4U and A4 40-cycle, immune selection resulting in structuring of *var* genes into discrete antigenic groups (Gupta and Anderson 1999), it was not expected to such a strong degree between the A4 isolates and either 3D7 or the clinical isolate P1. That this correlation was not simply explained by exposure was even more remarkable. This finding led to the hypothesis that perhaps what is being recognised was not a variant antigen but a conserved target leading to variant-transcending responses or at least a subgroup of cross-reactive or common epitopes within more diverse antigens. A degree of isolate specificity in antibody response is also apparent however, with the line of best-fit not dissecting zero indicating preferential recognition of one isolate over another (figure 3.8).

In order to dissect this response further, each isolate was pre-treated with trypsin. All currently identified surface exposed parasite antigens are known to be differentially trypsin sensitive (Leech, Barnwell et al. 1984; Sherman, Crandall et al. 1992; Kyes, Rowe et al. 1999; Winter, Kawai et al. 2005). It was thought that if the reactivity measured was not abrogated by trypsin pre-treatment, then perhaps a novel antigenic target for heterologous responses may be responsible for the tight correlations observed. As shown, reactivity to each isolate was sensitive to trypsin to varying degrees, with 3D7 significantly less sensitive than responses to A4. However the correlations were maintained after pre-treatment with 10 $\mu$ g/ml of trypsin which had abrogated all responses to either of the A4 isolates by more than 80%. It was only after pre-treatment with 1mg/ml when there was barely any reactivity to A4, that all correlations were lost. A previous study looked in detail at precisely the sensitivity of different epitopes within A4 *Pf*EMP1 to trypsin (Fagan 1999). A4 *Pf*EMP1 is sequentially cleaved by increasing concentrations of trypsin, from the N-terminal end with DBL domains  $\delta$ ,  $\gamma$  and  $\alpha$  to  $\beta$  remaining attached through inter-domain disulphide bonds even once the intervening sequence has been cleaved. This work also demonstrated the molecular target for the monoclonal antibody, BC6, lying within the inter-domain region between domains  $\delta$  and  $\epsilon$ . It is known that variant antigens, specifically *Pf*EMP1 differ in their sensitivity to trypsin (Gardner, Pinches et al. 1996). Also RIFINs appear to be less trypsin sensitive than *Pf*EMP1 (Kyes, Rowe et al. 1999).

It is difficult to conjecture what target might be being recognised to the same extent by each individual in this study however the data presented provide evidence of an isolate transcending response. It is not possible however to say conclusively whether



this response is to a relatively trypsin resistant component of *Pf*EMP1 or to another more conserved target. If a pan-reactive response or a variant-transcending target could be established on the surface of infected erythrocytes then the implications for vaccine design could be of immense importance.

It was with this aim that we went on to look in more detail at the domain-specific response to A4 *Pf*EMP1 domains. Prior to this, however we successfully cloned and expressed the domains of A4 *Pf*EMP1 as recombinant fusion proteins. The results of this set of experiments are outlined in chapters 4 and 5.

# Chapter 4

## Production of expression constructs and expression of recombinant proteins

### Introduction

The heterologous expression of recombinant protein has become a standard process in molecular biology. It has become essential for the characterisation of almost all proteins, from determination of antigenicity to functional analysis and vaccine development. Among the many systems available for recombinant protein production, the gram-negative bacterium *Escherichia coli* (*E. coli*) is one of the most attractive for reasons including its ability to grow rapidly and at high density, its well-characterised genetics and the availability of many types of expression systems tailored to individual requirements through the use of different cloning vectors and mutant strains of bacteria.

### Selection of expression system

In utilising a heterologous expression system, it is hoped that the resultant recombinant protein will mirror as far as possible, the native protein in terms of conformation and tertiary structure. This is particularly important if the resultant protein will be used in functional studies or as an antigen recognised by antibodies in human sera. The resultant recombinant protein and how much it resembles the native form is dependent on the correct folding pathway and appropriate post-

translational modifications occurring. Malaria antigens are amongst the most difficult proteins to produce *in vitro*. This stems from the fact their genome consists of a high number of A/T repeats. Many systems have been utilised for the production of malaria antigens including *E. coli* (Baruch, Pasloske et al. 1995; Baruch, Ma et al. 1997; Abdel-Latif, Khattab et al. 2002), baculovirus (Pang, Hashimoto et al. 2002), and yeast (Baruch, Gamain et al. 2002).

Using baculovirus as an expression system has resulted in the production of correctly folded proteins (Pizarro, Chitarra et al. 2003). However problems of toxicity have been encountered with many recombinant proteins being toxic to the insect cells. As a result of this the system achieves low yields of protein making it a rather inefficient. Recently expression of malaria proteins in yeast cells has been optimised (Baruch, Gamain et al. 2002) (Kocken, Withers-Martinez et al. 2002; Miles, Zhang et al. 2002; Pan, Huang et al. 2004). However the complicated manipulation of sequences required prior to protein expression and the relatively long cultivation time make it unlikely that this method will become standard, reviewed in (Chang 1994).

As mentioned, the *P. falciparum* genome is extremely A/T-rich in terms of nucleotide composition. This complicates the expression of *P. falciparum* proteins in heterologous systems as the resultant abundance of lysine and arginine residues in the expressed proteins results in them being positively charged thus hindering their expression in *E. coli*. The high A/T content of the mRNA template also causes early termination of the protein and can be responsible for heterogeneity in the final expressed protein product. In using the *E. coli* system, this has meant expression

can be limited by the rarity of tRNAs in *E. coli* present in abundance within the *P. falciparum* genome (Yadava and Ockenhouse 2003). This can be circumvented by using strains of *E. coli* which have been transfected with a plasmid carrying extra copies of those tRNAs in short supply.

There have been many examples of successful expression of malaria proteins from bacterial systems either directly or in combination with a fusion protein (Baruch, Pasloske et al. 1995; Baruch, Ma et al. 1997; Fagan 1999; Abdel-Latif, Khattab et al. 2002). Also, there have been no reports of significant post-translational modification to any domain of PfEMP1. In eukaryotes the principal modifications can include glycosylation, where carbohydrate molecules are added to the protein structure, cleavage, phosphorylation and methylation. These previous successful attempts at cloning coupled with the apparent minor role of post-translational modification in the expression of *P. falciparum* proteins led us to use a bacterial expression system to express the domains of PfEMP1 in this study.

### **Selection of expression vector**

Many current techniques for bacterial expression utilise fusion proteins. Examples include glutathione-S-transferase (GST) (Smith and Johnson 1988), maltose-binding protein (MBP) (di Guan, Li et al. 1988) and a simple repeat histidine tag (Stuber 1990). Although fusion proteins were originally designed to facilitate protein purification and immobilisation, it has become apparent that certain fusion proteins greatly improve the solubility of the protein of interest. How this happens is not entirely clear although it does seem to depend on the order of protein synthesis, with

importance placed on the fusion protein being synthesised first (Sachdev and Chirgwin 1998). Not all fusion proteins are equally proficient at increasing solubility, in a study comparing the effectiveness of various fusion partners MBP was found to be far superior to alternative fusion protein systems including GST, as a solubilising partner (Kapust and Waugh 1999).

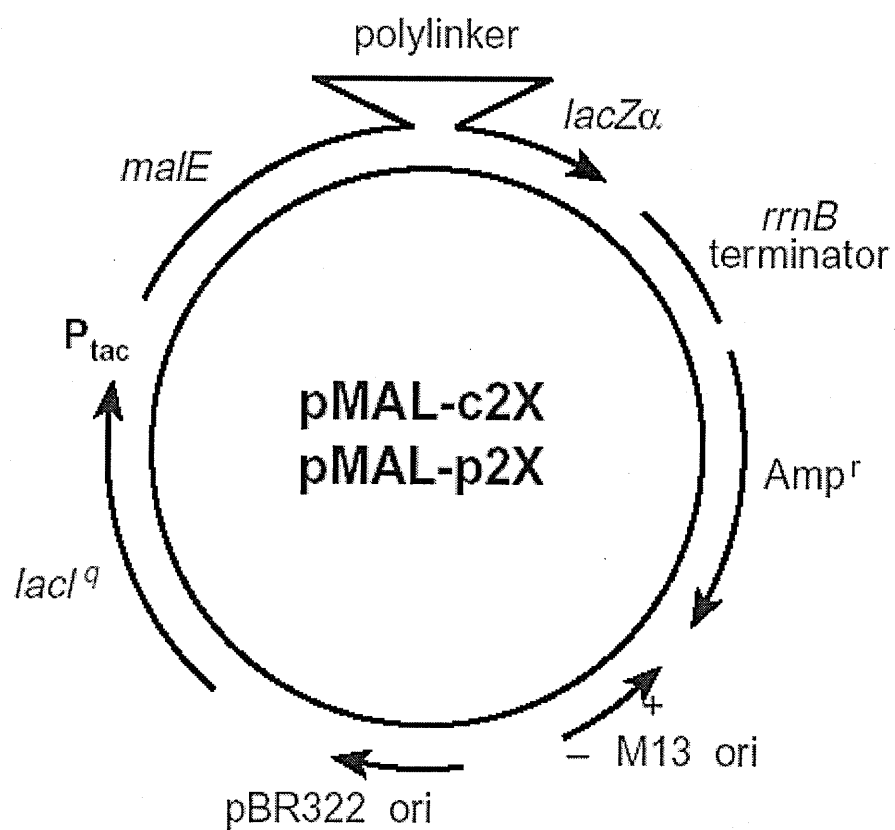
This information as well as the fact that GST is derived from *Schistosoma japonicum* (the population under study may have been exposed to *Schistosoma haematobium* which could potentially have a degree of antigenic similarity to *Schistosoma japonicum*) led us to conclude that the expression vector MBP was the optimal vector for use in these studies.

First devised in 1988, (di Guan, Li et al. 1988; Maina, Riggs et al. 1988), the pMAL-2 vector system allows a cloned gene to be inserted downstream from the *malE* gene of *E.coli*, which encodes MBP, producing a recombinant protein fused to MBP. This vector system utilises the *tac* promoter and the *malE* translation initiation signals to give high-level expression of the fusion protein, (Duplay, Bedouelle et al. 1984; Amann and Brosius 1985) as well as allowing simple purification through the affinity of MBP for maltose, (Kellermann and Ferenci 1982). The vector carries the *lacI<sup>q</sup>* gene, which codes for the lac repressor. This keeps expression low in the absence of isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) induction. The inclusion of an ampicillin resistance gene allows for selection of transformed bacteria (figure 4.1).

## Selection of domains for cloning

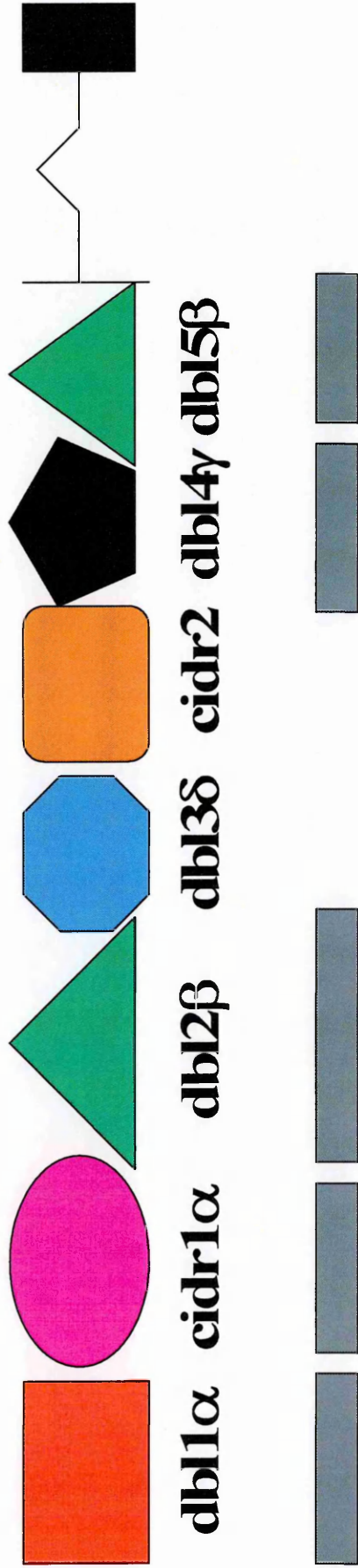
As described, within the *P. falciparum* genome there are around 60 copies of a gene family called *var*, coding for proteins known as *PfEMP1* (Gardner, Hall et al. 2002). The *var* genes are made up of three distinguishable domains known as Duffy binding like domains, (DBL), cysteine rich interdomain regions, (CIDR) and the conserved exon 2 (Peterson, Miller et al. 1995; Baruch, Ma et al. 1997; Smith, Subramanian et al. 2000). Although each variant shares this common structure they are polymorphic in terms of sequence. From sequence alignments these domains have been characterised into different sub-classes, for the DBL domains  $\alpha$  to  $\epsilon$  and for the CIDR domains  $\alpha$  to  $\gamma$ . Figure 4.2 outlines a schematic of one of these genes, the A4 *var* gene showing the domains cloned and expressed in this project.

Experiments were conducted using *P. falciparum* parasites of the laboratory adapted A4 lineage. Sequential cloning from the Brazilian line IT 4/25/5, a gift to Dr C I Newbold from Dr R Howard, derived this line; figure 4.3 illustrates its derivation. Domain sequences were amplified from the parasite line A4 and corresponded to the sequence of A4 *PfEMP1*. This particular variant of *PfEMP1* was chosen for a number of reasons. It has a well-described and classified sequence already published with established domain boundaries. It displays a common cytoadherent phenotype, binding to both CD36 and ICAM-1 and is a useful tool in the laboratory as there exists a monoclonal antibody, BC6, specific for this variant, allowing *in vitro* selection of A4 parasites expressing specifically A4 *PfEMP1* (Smith, Chitnis et al. 1995; Smith, Subramanian et al. 2000), and lastly previous work undertaken in the area of Kenya where these studies were done highlighted recognition of this strain of



**Figure 4.1**

Map of pMAL-c2X vector (also sister vector pMAL-p2X which differs in that the malE signal sequence is maintained allowing export of the recombinant protein to the cellular periplasm if required), note the pMAL -c2X vector includes an exact deletion of the malE signal sequence.



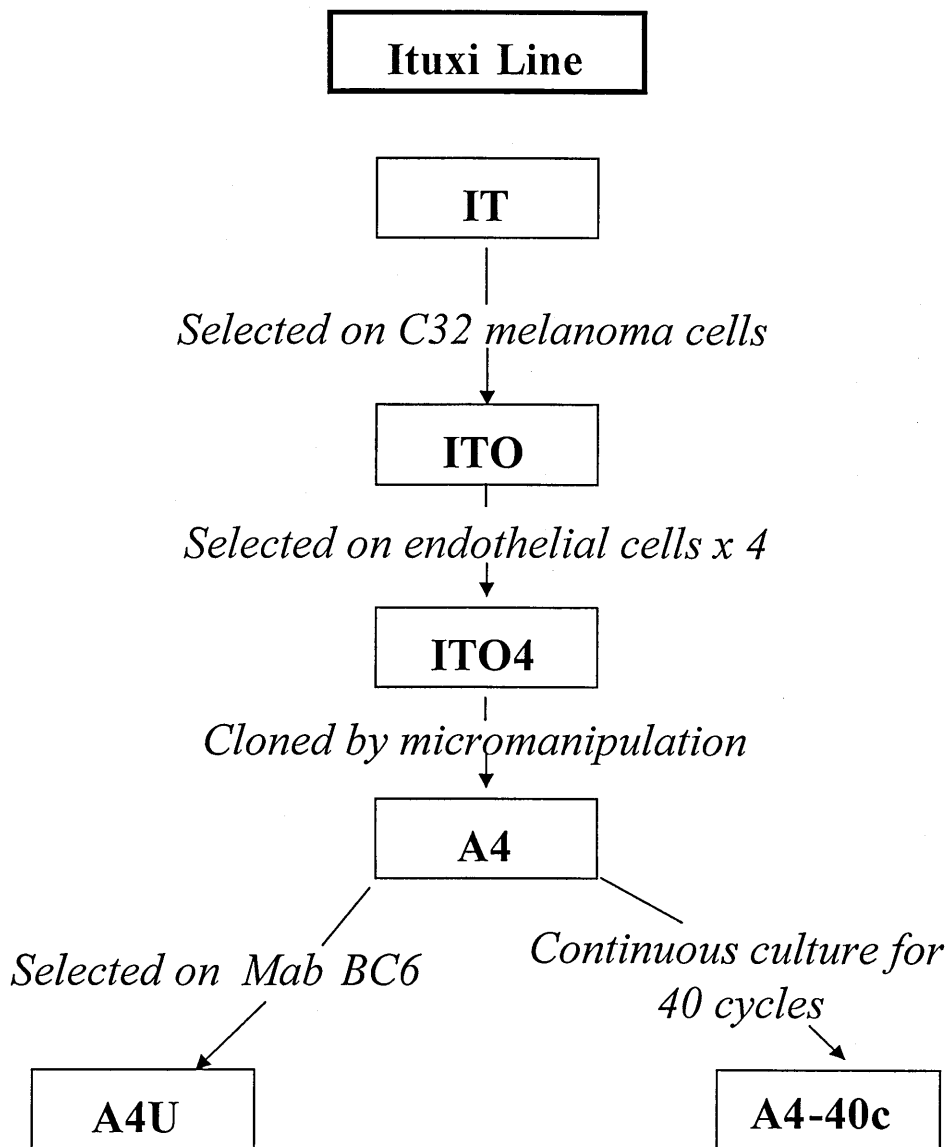
**Figure 4.2**

### **A4 *var* gene**

Schematic of the A4var gene. The coloured shapes correspond to individual extracellular variant domains and the black rectangle illustrates the conserved intracellular exon

2. The grey boxes illustrate those domains expressed as recombinant proteins in this thesis.





**Figure 4.3**

**Derivation of the A4 laboratory parasite line (Roberts, Craig et al. 1992)**

The Ituxi parasite line is a knob-forming, cytoadherent strain originating in Ituxi, Brazil. Mab BC6 is a monoclonal antibody specific for the major PfEMP1 variant expressed on the surface of erythrocytes infected with late stage A4 parasites, A4 PfEMP1. A4U (A4 ultra) are parasites selected for expression of A4PfEMP1 through use of Mab BC6 and A4-40c are parasites, which have been in culture for 40 cycles of growth with no immune selection.

cloned parasite by around 70% of adults and 30% of children as measured by agglutination (K. Marsh *unpublished data*).

The intention was to express each domain of A4 *Pf*EMP1 including the cysteine rich interdomain region (CIDR). A4 genomic DNA, kindly supplied by Sue Kyes, Oxford, was used as a template for cloning. Primers were designed spanning the domain and interdomain boundaries as outlined in the published A4 sequence (Smith, Kyes et al. 1998), with restriction enzyme sites attached to allow directed cloning into the multiple cloning site of p-MALc2x vector. Sequences of the primers used are given in table 4.1. Initially it was felt that separate and 3' from DBL2 $\beta$  was a conserved cysteine rich region called C2. In the original cloning experiments this region was not included in the primer design or eventual protein expression. DBL5 $\beta$  has also a region 3' to it which is cysteine rich although not classified as a C2 region. This region again was not initially included in the primer design. As information became available that these areas were to be classified as part of the  $\beta$ -type domains primers were re-designed and the resultant proteins re-expressed {Chris Newbold and Sue Kyes *personal communication*}.

### **Optimisation of PCR**

PCR reactions were carried out to amplify selected regions of A4 *Pf*EMP1 DNA for cloning. Optimisation experiments were performed prior to using any set of primers using variations of thermal cycling times, magnesium chloride concentrations, template, and primer concentrations (Harris and Jones 1997; Fagan 1999). Optimisation focused on obtaining the exact stringency required to amplify the

region of interest over competing regions of other *var* genes. Table 4.2 demonstrates the final cycling conditions used. Yields varied between 0.5 and 5 $\mu$ g DNA per 50 $\mu$ l reaction. Note that after PCR using the primers specific for domain db138, it became clear from the size of the DNA amplified that the region cloned using these primers was too large. Detailed analysis of the A4 *var* sequence revealed an intron, which had mistakenly been incorporated within the primer boundaries. The reverse primer for this domain was thus re-designed and the cloning process re-started. Unfortunately no protein was successfully expressed using these primers and the attempt was abandoned.

**Table 4.1**

**Primer sequences used to amplify the sections of DNA corresponding to each desired region of the protein.**

Name	Primers	Primer Sequence 5' to 3'
A4 dbl1 $\alpha$	A4 dbla SmaI F	GCTCCCCGGGGGTCATGGTAGGGAGCATCCT
	A4 dbla1 HindIII R	CCCCAAGCTTGCCATATCCGTATGAGAAAATG
A4CIDR1 $\alpha$	A4 CIDR1 SmaI F	GCTCCCCGGGGCAGGTGGATTATGTATATTCG
	A4 CIDR1 PstI R	CCCCTGCAGCTATGAATCACCAATAGCATTGG
A4 dbl2 $\beta$	A4 dblb SmaI F	GCTCCCCGGGACGAACCAATATCCAATGC
	A4 dblb XbaI R	CCCCTCTAGACTACCACCCCCAATGTCGTTGTGG
A4dbl2 $\beta$ c2	A4 dbl2bc2 XbaI R	CCTCTAGACTAGCACACATCCAACCTGGTGTC
A4 dbl4 $\gamma$	A4 dbld SmaI F	GCTCCCCGGGTGCAATACAAAATATTATCCAAC
	A4 dbld HindIII R	CCCGCAAGCTTGCTACGAAGCAAATGTACTGTC
A4 dbl5 $\beta$	A4 dble1 SmaI F	GCTCCCCGGGGCTTCGAATTGTGAAC
	A4 dble1 HindIII R	CCCGCAAGCTTGCTAACGACGTGCAGTGCTAGG
A4 dbl5 $\beta$ c	A4 dbl5b+C XbaI R	CCTCTAGACTAGATTTCGGATCGTTATTACTCG

Restriction sites are identified in bold type. Primers named F follow the 5' to 3' coding sequence of the DNA and those denoted R were reverse primers specific for the anti-sense strand. Note for Dbl2 $\beta$  and Dbl5 $\beta$  there are two reverse primers, the first one was used initially before the realisation of c2 being part of the  $\beta$ -type domain and the second primer was ordered later to incorporate this cysteine-rich area as outlined above.

**Table 4.2**

**The final cycling conditions used for the PCR outlined for each domain cloned.**

Region	MgCl <sub>2</sub> Conc.	Temp °C	Time/mins	No. of Cycles
A4 db11 $\alpha$	3.5mM	94	4	1
A4 CIDR1 $\alpha$	2.5mM	94	0.5	35
A4 db12 $\beta$	2.5mM	50	0.5	
		65	2	
		65	5	1
A4 db14 $\gamma$	2.5mM	94	4	1
A4 db15 $\beta$	3.5mM	94	0.5	35
		55	0.5	
		65	2	
		65	5	1

## **Cloning**

DNA was purified from PCR reactions as previously described. Following treatment with the appropriate restriction enzymes, DNA inserts were again purified by electrophoresis and agarose digestion again as described. Inserts were ligated into the p-MALc2x vector, which had been previously cut with the appropriate restriction enzymes and undergone treatment with calf intestinal phosphatase to prevent self-ligation. The resulting plasmid was transformed initially into DH5 $\alpha$  strain *E. coli*. Ampicillin selection was applied to identify transformed colonies of bacteria.

## **Screening of Recombinant Plasmids**

Plasmid extractions were performed, as described, on clones derived from initial transformation reactions. Restriction digests were performed on the purified plasmid DNA to determine if an insert of the correct size was present. Digests were done using the specific restriction enzymes attached to each primer as described in table 4.1. A representative sample of digests is illustrated in figure 4.4.

Once the presence of a correctly sized insert had been confirmed, the plasmids were transformed into TB1 or latterly BL21-CodonPlus-Ril bacteria cells for expression. These cells were grown on LB media plates supplemented with both chloramphenicol and ampicillin, for BL21-CodonPlus-Ril cells and ampicillin alone for TB1 cells, as described. To further check presence of a correctly sized insert, colonies grown on such plates were screened by PCR using p-MALc2x specific primers. The forward primer initiates at the 3' end of the *malE* gene around 81bp upstream of the primary site in the polylinker. The reverse primer is initiated within

the lacZ $\alpha$  gene. The details of these primers are given in table 4.3. The PCR screening was undertaken by lifting an isolated colony from the overnight growth plate and adding it briefly to 24 $\mu$ l of PCR buffer containing the p-MALc2x specific primers before adding the colony definitively into a 1ml aliquot of LB medium. The PCR was run with the cycling times outlined in table 4.4 and the culture was incubated at 37°C until the end of the PCR run. Those colonies that were positive for the presence of the insert were grown overnight in LB supplemented with ampicillin and chloramphenicol or ampicillin alone, shaking at 37°C before being stored at – 20°C in 20% glycerol.

### **Initial Expression Screening**

Transformed bacteria were checked for successful expression of recombinant proteins following plasmid screening. This verified that premature stop codons had not been introduced by PCR errors and checked different clones containing the same constructs for relative levels of expression. To verify expression of the recombinant protein, non-induced cultures were treated and analysed for expression in parallel with cultures that had been induced by IPTG.

Initial results showed adequate expression of all constructs when assessed crudely. There were variations amongst individual clones and between domains with CIDR1 $\alpha$  proving the most difficult to express at high enough yields. Examples of crude expression of all the constructs is shown in figure 4.5.

**Optimisation of Preparative Scale**

Once a clone had been identified as an efficient expressor of recombinant proteins large scale preparations were performed. Several factors were found to influence yield and these were individually investigated for each protein. Yields varied between proteins and ranged from 0.1mg/ml to 2mg/ml of final eluate. Factors found to influence yield included temperature at induction, expression time, strain of bacteria used for expression, method of cell lysis, and concentration of maltose used in elution. Bulk preparations of protein were done using culture volumes of between 1 and 2 litres, optimisation experiments were carried out on smaller volumes of 50ml. Adequate amounts of all the domains were obtained once the elution conditions were optimised as described, CIDR1 $\alpha$  however required more extensive optimisation before adequate yields were obtained and this will be described.

**Table 4.3**

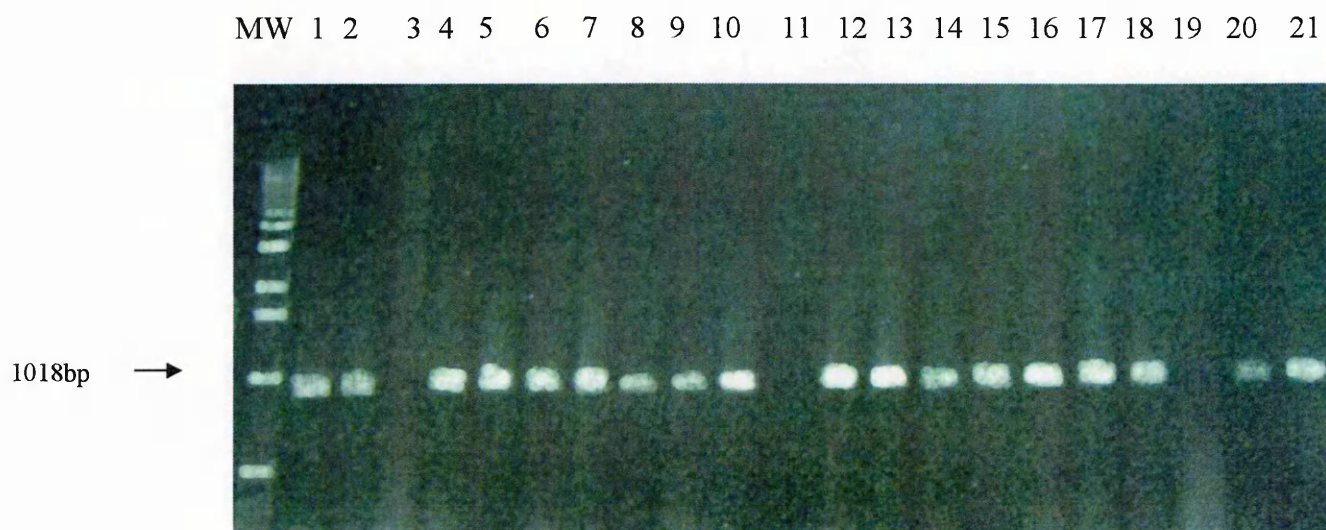
**p-MALc2x specific primers**

Primer region	Sequence 5' to 3'
p-MALc2x male F	GGTCGTCAGACTGTCGATGAAGCC
p-MALc2x M13 R	GTAAAACGACGGCCAGT



**Table 4.4**  
**PCR cycling times for screening transformed colonies**

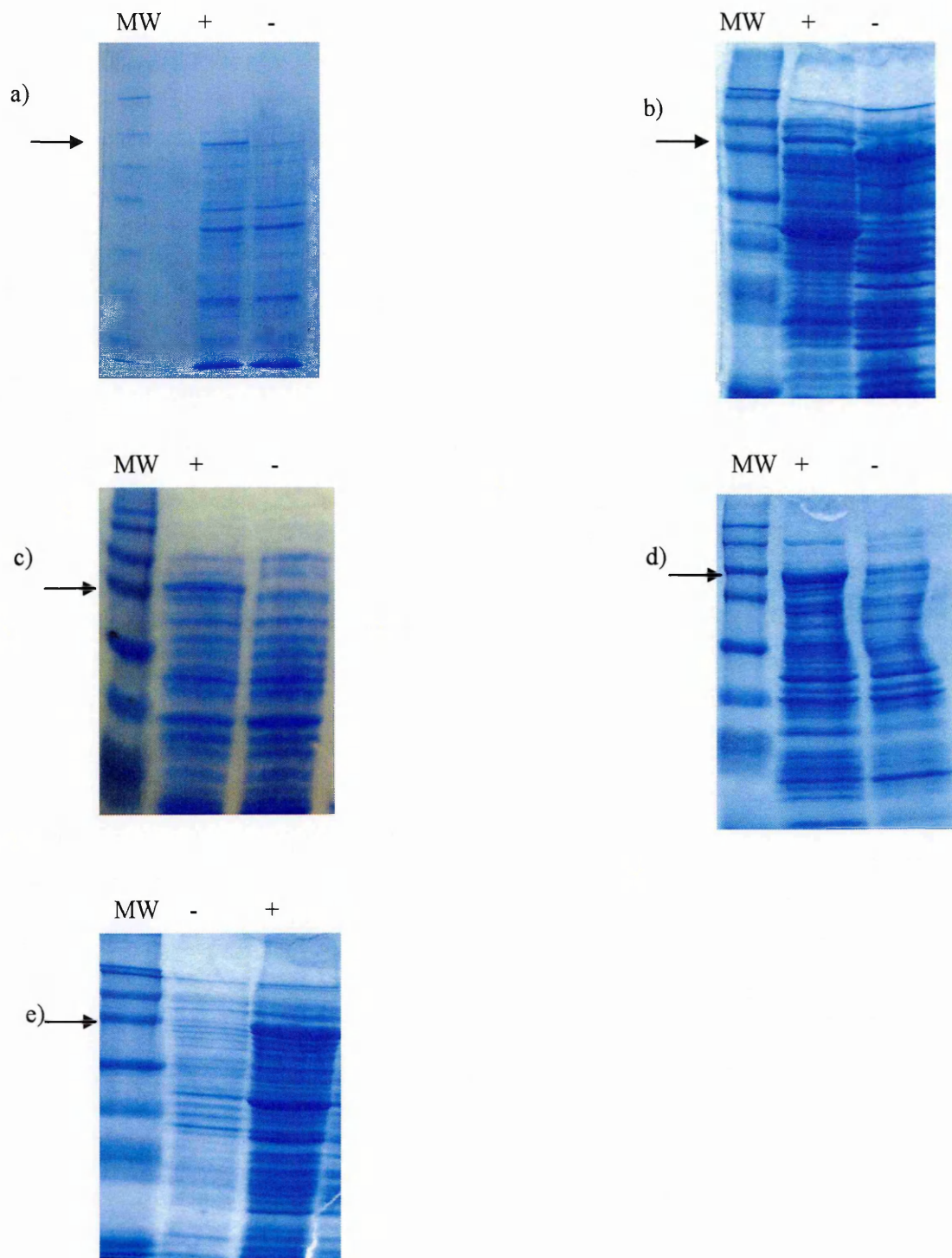
Temp °C	Time/Mins	No Of Cycles
95	1	1
94	0.5	35
50	0.5	
65	1.5	
65	5	1



**Figure 4.4**

**An example of PCR screening for correctly sized inserts**

An example of screening transformed bacteria containing the insert CIDR1 $\alpha$  (761bp including MBP fusion) using the p-MALc2x specific primers. As is obvious colonies 3, 11 and 19 did not show any evidence of containing a correctly sized insert.



**Figure 4.5**

Gel showing comparison of IPTG induced (+) and uninduced (-) expression of MBP fusion proteins.

MW molecular weight ladder a) DBL1 $\alpha$ , b) DBL2 $\beta$  c) CIDR1 $\alpha$  d) DBL4 $\gamma$  e) DBL5 $\beta$ . The arrows indicate the intact fusion proteins.

### **Elution conditions**

Overall the most important factor influencing yield was the concentration of maltose used to elute the recombinant protein from the amylose resin column. In order to optimise elution conditions, 50 ml cultures were grown induced and cells lysed as described previously. To the bacterial lysate obtained after sonication, 500µl of pre-equilibrated amylose resin was added and the tubes rolled at room temperature for 1 hour. The lysate and column was spun briefly and the supernatant removed, 2mls of column buffer was then added to the column beads and the beads were rolled for 1 minute and the supernatant removed each time. The column beads were then split into 5 x 20µl amounts of amylose resin and to each was added elution buffer with a different concentration of maltose added (100mM, 50mM, 30mM, 20mM, 10mM). Each eppendorf tube containing the column beads and elution buffer was then rolled for 30 minutes, centrifuged briefly and the supernatants collected. Protein elution was then assessed on a 10% SDS-PAGE gel. Figure 4.6 illustrates elution of CIDR1α as an example of the effect of increasing the maltose concentration in the elution buffer.

### **Temperature**

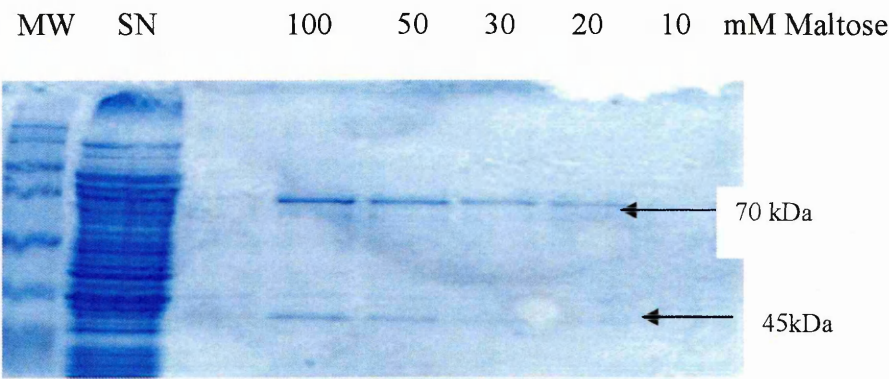
Growth at low temperature has been found to improve the yields of expression constructs in heterologous systems.(Airenne and Kulomaa 1995; Yu, Deng et al. 1995) The effects have been reported as both reducing degradation of soluble proteins and reducing aggregation and resultant insolubility. It was consistently difficult to obtain soluble CIDR1α with intense bands corresponding to the size of the intact fusion protein present within the insoluble pellet suggesting that

considerable amounts of the protein aggregate within the bacterial cytoplasm. An example of the insoluble fraction of this particular protein is illustrated in figure 4.7. Induction temperatures of 30°C, 25°C and 20°C were compared to 37°C and it was found that all temperatures lower than 37°C lessened this problem considerably with 25°C giving optimal balance between improved yield and lengthened incubation time, figure 4.8.

### Strain of bacteria

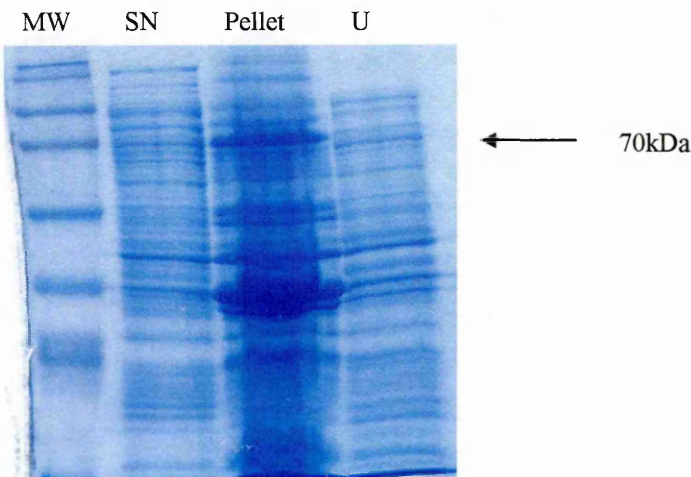
Bacterial strain DH5 $\alpha$  was used for all cloning steps and initial protein expression. Following this initially, *E. coli* strain TB1 (F<sup>-</sup> *ara*  $\Delta$ (*lac-proAB*) [ $\Phi$ 80*dlac*  $\Delta$ (*lacZ*)M15] *rpsL*(Str<sup>R</sup>) *thi hsdR*) was used for large scale expression. However, expression of all the fusion proteins proved extremely unstable in this system and this was thought due to the fact this strain was not protease deficient. Of additional concern when cloning and expressing from *P. falciparum* DNA is the high A/T content of the genome. As mentioned, the paucity of tRNAs necessary to cope with such a nucleotide composition can limit expression of the full-length protein. BL21-codonplus-Ril cells were then compared for expression efficiency and found to be superior. This strain of *E. coli* naturally lacks the *lon* protease, which can degrade recombinant proteins and also has extra copies of the *argU*, *ileY* and *leuW* tRNA genes in the form of a pACYC plasmid. These genes encode tRNAs that recognise the arginine codons AGA and AGG, the isoleucine codon AUA and the leucine codon CUA respectively, together the tRNAs that most frequently restrict translation of heterologous proteins from organisms with AT-rich genomes.

**Figure 4.6**



Gel showing CIDR1 $\alpha$  fusion protein obtained with different concentrations of maltose in the elution buffer. Note progressively more protein was obtained with higher concentrations. SN is the bacterial lysate after rolling with the columns for 1 hour as described, 70kDa is the predicted size of the CIDR1 $\alpha$  fusion protein. 45KDa is the predicted size of MBP, the fusion partner.

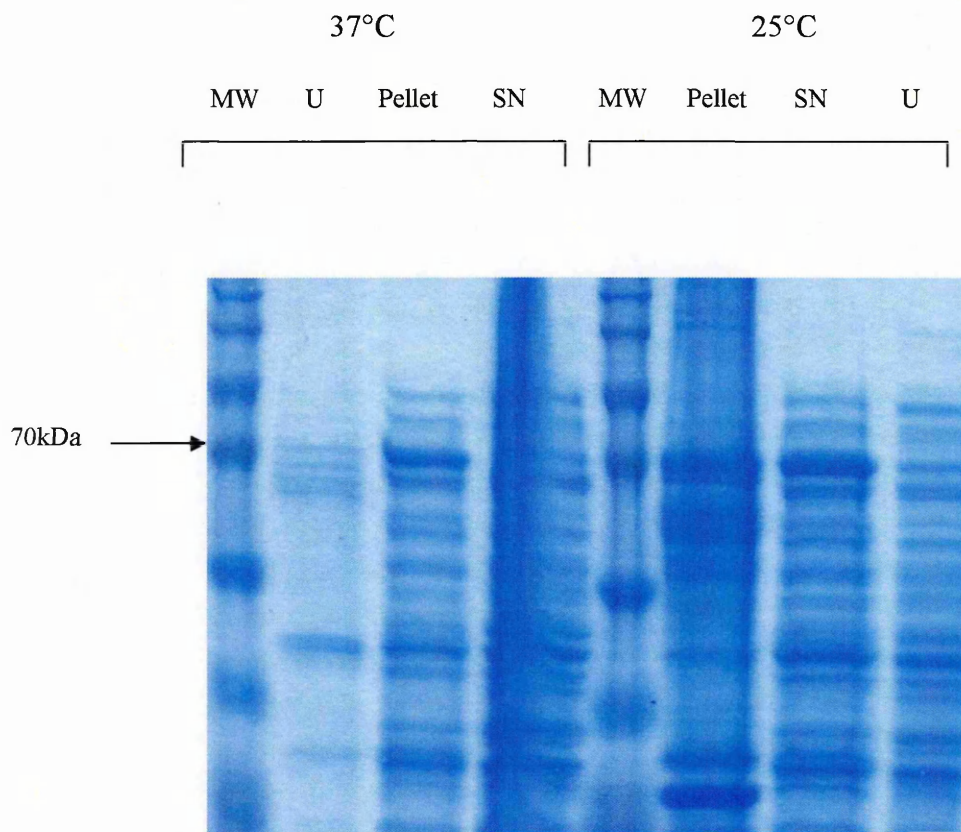
**Figure 4.7**



Gel showing the majority of expressed CIDR1 $\alpha$  present within the bacterial pellet.

MW molecular weight ladder, SN supernatant and U uninduced culture. 70kDa is the predicted size of CIDR1 $\alpha$ .





**Figure 4.8**

Gel illustrating the effect of lowering the induction temperature from 37°C to 25°C. MW molecular weight ladder, SN supernatant, U uninduced, 70kDa is the predicted size of the CIDR1α fusion protein.

### **Summary of expression experiments**

Final purified products of all the recombinant proteins are shown in figure 4.9. Many proteins suffered from some premature termination or degradation. Assessment of the concentration of the proteins was made using two methods. For crude estimates of concentration, samples of purified protein were compared on an SDS-Page gel to known amounts of bovine serum albumin (BSA). The relative intensity of the coomassie stained bands gave an approximate value for the protein concentration of the sample.

For more accurate quantification, a soluble phase protein assay was carried out using a Protein Assay kit (Bio-Rad). Coomassie Blue was used to stain proteins in aqueous solution with results being read by spectrophotometry at 595nm. BSA was used as a standard.

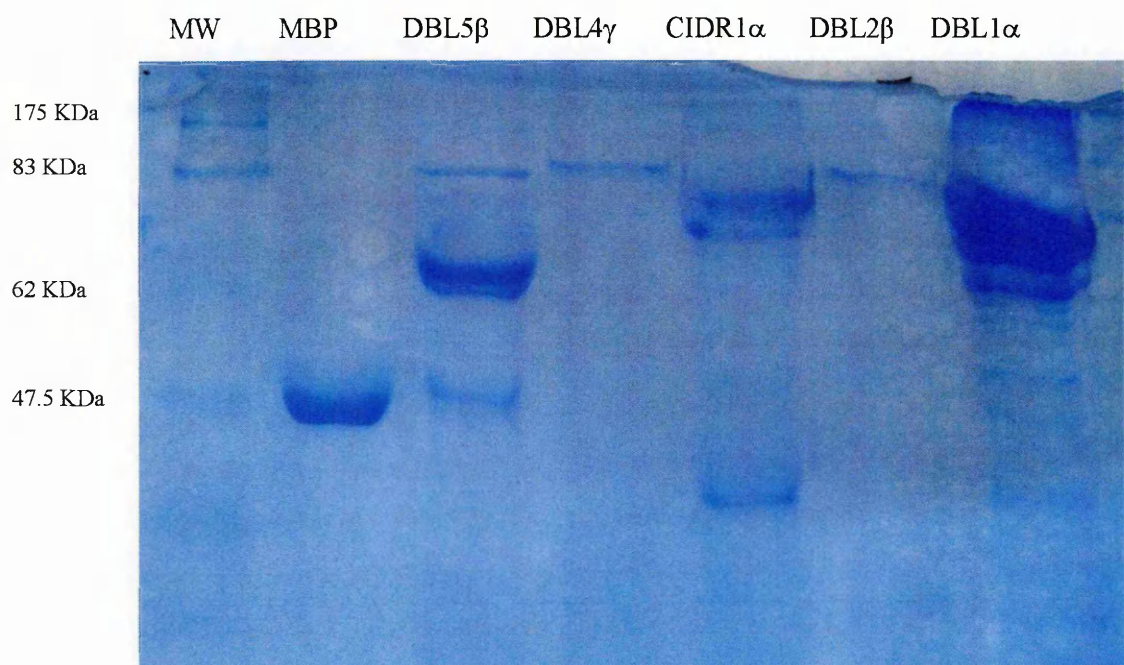
Both of these techniques depended on the relative dye binding properties of BSA against the experimental samples. These will vary between individual proteins. Thus, the results of these assays can only be expressed as values relative to BSA.

### **Sequencing**

Sequencing was performed on the junction sites of the DNA constructs as they were produced. This was done using manual sequencing techniques and sought to confirm that a correct reading frame had been generated and that the initial sequence



was correct. This was confirmed for all constructs. Later experiments to verify the entire insert sequence were performed using an automated sequencing method. Sequence reactions were repeated by the Department of Biochemistry, University of Oxford.



**Figure 4.9**

### **Expression of all domain constructs**

Gel showing expression of all the PfEMP1 domains expressed. On the right hand side are the protein size markers. As some bands are extremely faint the corresponding molecular weight is marked for comparison. The domains are labeled across the top of the gel with lane one showing expression of MBP alone.

The expected sizes of the fusion proteins are as follows:

DBL1 $\alpha$  – 90KDa

DBL2 $\beta$  – 90KDa

CIDR1 $\alpha$  – 70KDa

DBL4 $\gamma$  – 90KDa

DBL5 $\beta$  – 80KDa

MBP – 45KD

## Discussion

Maltose-binding protein would appear to be a successful tool for the expression and purification of recombinant proteins. Significant problems were met with when eluting the protein in a soluble form, whereas expression of the construct itself was efficient. The yield obtained using this system, while adequate is not massive with some of the constructs proving easier to purify than others. The CIDR1 $\alpha$  domain proved most difficult to purify, with the majority of the protein expressed remaining insoluble after bacterial cell lysis. Many strategies were employed to deal with this, though only inducing the protein at a lower temperature made any difference. All purification was aided by eluting at a higher maltose concentration than the manufacturers recommend.

It is unknown to what degree recombinant proteins must retain their native structure in order to raise antisera against them. Previous work has indirectly shown that antisera raised against bacterially expressed recombinant proteins can, in one case inhibit the function of native *Pf*EMP1 (Baruch, Pasloske et al. 1995), and in another study recognise the surface of intact A4 parasitised red blood cells (Fagan 1999). The hope in this thesis was that these recombinant proteins would retain tertiary structure enough to measure domain-specific antibodies present in the sera of individuals resident within a malaria endemic area.

# Chapter 5

## Naturally acquired antibody responses to recombinant protein domains of *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP1)

### Introduction

Having successfully expressed recombinant proteins covering major domains of A4 PfEMP1, each domain was characterised with regards to its recognition by sera collected from adults and children resident in two areas of different *P. falciparum* transmission intensity. The intention was to describe overall patterns of response to each domain and how they alter with age and transmission. A further aim was to describe individual variation of recognition between individuals and between domains. In order to demonstrate that responses to the recombinant domains were of importance in vivo, the correlation of responses to each domain with those to the surface of A4-infected erythrocytes were measured. Finally I attempted directly to purify anti-domain specific antibodies.

### Methods

Recombinant proteins corresponding to the major domains of A4 PfEMP1 were expressed and purified as described in Chapter 4. Reactivity was measured against each domain using an ELISA method previously optimized as described in Chapter 2. After obtaining informed consent, sera from 1,222 asymptomatic individuals were obtained at a cross-sectional bleed in Chonyi and Ngerenya in October 2000 as part

of an on-going study (Mwangi 2003). The baseline characteristics of the individuals tested are summarised in table 5.1. A blood slide was taken at the time of bleed and later analysed for the presence of detectable parasites by microscopy. Individuals were followed up by weekly active malaria case detection and continuous passive case detection. A case of malaria was defined as:

Individuals aged less than 1 year – fever  $> 37.5^{\circ}\text{C}$  plus any parasitaemia

Individuals aged over 1 year – fever  $> 37.5^{\circ}\text{C}$  plus parasitaemia  $> 2500/\mu\text{l}$  (Mwangi 2003)

All ELISAs were conducted in duplicate and if a disparity greater than 15% existed between the two readings, they were repeated, again in duplicate. In order to standardise readings between plates a previously defined positive control serum sample was included on each plate in duplicate as well as 5 non-malaria exposed donor sera, obtained from Oxford, UK. Sera were tested blind to the age and parasite status of each individual, although the location, Ngerenya or Chonyi, was known. All sera were tested against each domain in a random order. All ELISA data was entered into an Excel spreadsheet and the average of duplicate wells taken. An individual was scored as antibody positive if the average OD obtained was greater than the mean plus 3 standard deviations of a panel of 20 non malaria-exposed donors. This cut-off was chosen in order to fully capture the variability which existed in reactivity shown by the non malaria-exposed donors in response to some domains, notably CIDR1 $\alpha$ . As part of a study investigating the relationship between malaria disease and genetic mechanisms of resistance, (Dr Thomas Williams) a proportion of samples were genotyped for  $\alpha$  thalassaemia status (normal,

heterozygote or homozygote) and for the presence of sickle cell trait (HbAS). All genotyping was performed by Dr. Williams, Mr Alex Mwacharia and Mr Sammy Wambua at the Centre for Geographic Medicine Research Coast.

### *Statistical analysis*

To investigate the relationship between parasite status at the time of cross-sectional bleed and subsequent antibody levels, a multiple linear regression model, controlled for age, expressed as a factor of 6 months duration, location and exposure was performed. The results are expressed as coefficients describing the difference in antibody levels attributable to parasites. The odds of being antibody positive if parasite positive were calculated using a multiple logistic regression model with age and location as co-variables. Significant differences in two or more continuous variables were calculated using the Wilcoxon rank sum test. The Chi-squared test for trend was used to assess a trend across groups.

In a randomly selected subgroup of individuals, total IgG responses to the intact A4 parasitised erythrocyte were measured using flow cytometry (see Chapter 3, table 3.1 for details of individuals tested). The parasite line A4U (kindly supplied by Mr Bob Pinches) was used. This clone of parasite expresses on its surface one dominant *var* gene, A4 through selection with the monoclonal antibody BC6 (Smith, Chitnis et al. 1995). The correlation of responses to each individual domain with responses to the parasitised erythrocyte surface was compared using the Spearman rank correlation coefficient.

Table 5.1 Baseline characteristics of individuals at cross-sectional survey

Location	Age (years)							Sex		Microscopically detectable parasites		$\alpha$ thalassaemia genotype			sickle cell genotype	
	0-3	3-6	6-10	10-15	15-30	>30		male	female	present	absent	$\alpha\alpha/\alpha\alpha$	$\alpha-/aa$	$\alpha-/a-$	HbAA	HbAS
Chonyi	103	90	105	92	97	114		244	357	219	377	77	109	32	not typed	not typed
Ngerenya	100	114	99	113	101	94		277	343	148	469	96	153	49	389	50
Total	203	204	204	205	198	208		521	700	367	846	173	262	81	389	50

In order to further clarify whether the responses measured by ELISA to each recombinant protein domain reflected domain-specific responses as may occur towards the intact erythrocyte surface expressed *Pf*EMP1, an attempt was made to purify the domain specific antibodies from sera of individuals. The aim was to assess whether or not these responses reacted against the surface of the intact A4 parasitised erythrocyte through flow cytometry. Domains DBL1 $\alpha$  and DBL4 $\gamma$  were selected because of the positive correlation between individual responses to these domains with individual responses to the intact parasitised erythrocyte. All statistical analysis was performed using Stata version 8 (Statacorp, TX, USA).

## Results

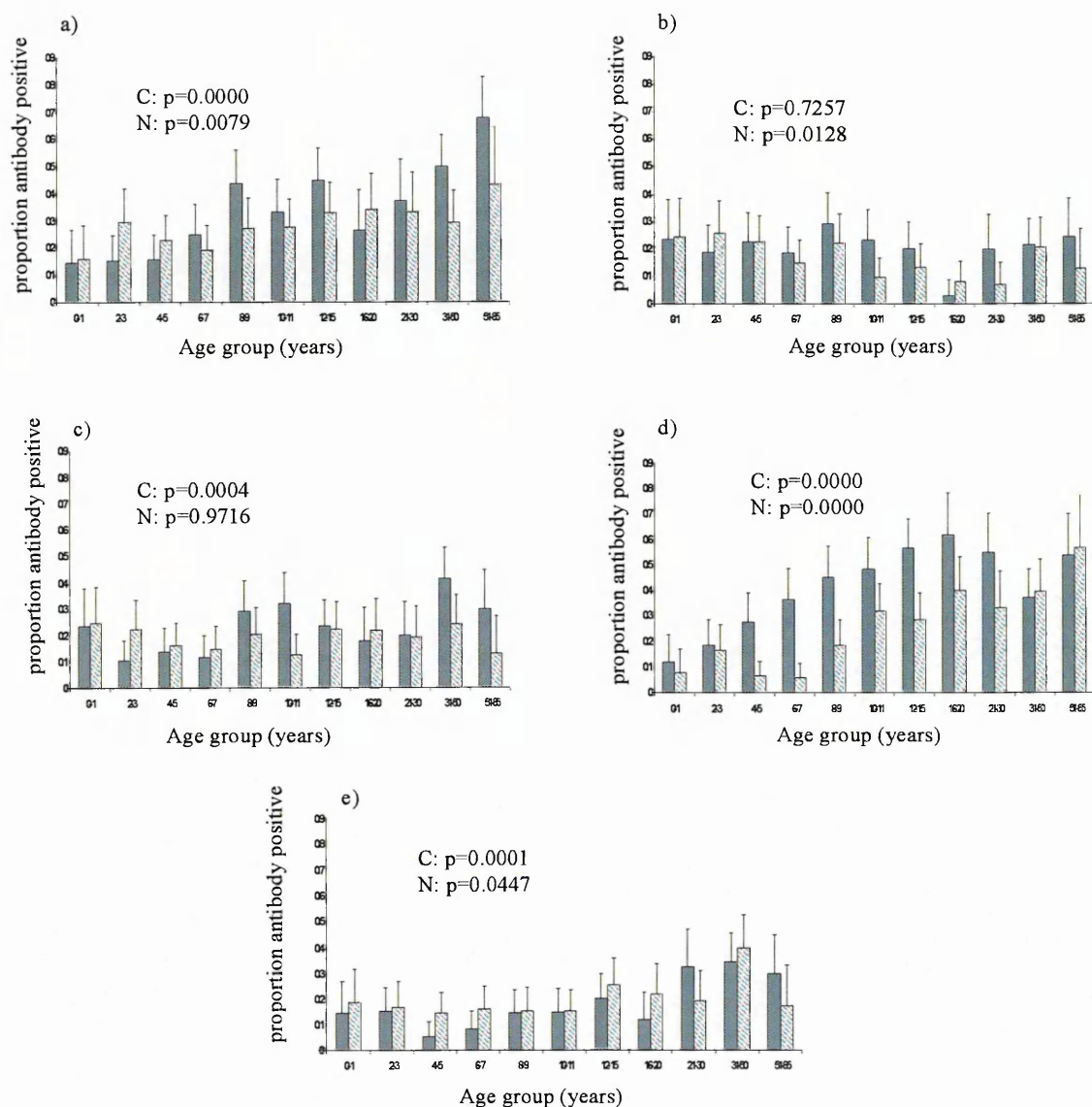
Sera from a total of 1222 individuals were tested by ELISA against five recombinant proteins corresponding to the DBL1 $\alpha$ , DBL2 $\beta$ , CIDR1 $\alpha$ , DBL4 $\gamma$ , and DBL5 $\beta$  domains of A4 *Pf*EMP1. Individual patterns of response and overall acquisition of antibodies to each domain was assessed.

### *Naturally occurring antibody responses to recombinant PfEMP1 domains*

Evidence for age-associated acquisition of domain-specific IgG was apparent for all domains, except CIDR1 $\alpha$  and DBL2 $\beta$ , in both Chonyi and Ngerenya (Figure 5.1). However, it is important to note that when the Chi-squared test for trend was applied only to the age ranges over which there is appreciable acquisition of anti-malaria disease immunity, ages 6 months to 5 years in Chonyi and ages 6 months to 10 years in Ngerenya, there was no significant age-associated trend of antibody acquisition, although this study was not designed in order to detect such differences. No



appreciable increase in antibody acquisition with age was seen with CIDR1 $\alpha$ , in fact in Ngerenya a significant loss of response with age was seen. Acquisition of antibody responses to DBL2 $\beta$  was only seen in the higher of the two transmission areas, Chonyi. Noticeable differences could be seen in the response to the different PfEMP1 domains. For example it is clear that a greater proportion of individuals recognise DBL1 $\alpha$  and DBL4 $\gamma$  than the other three domains with overall 30.8% (95% CI 26.2 – 35.8%) and 32.5% (95% CI 27.9 – 37.3%) of individuals recognising DBL1 $\alpha$  and DBL4 $\gamma$  respectively, compared to 18.8% (95% CI 13.9 – 24.3%), 21.2% (95% CI 16.4 – 26.7%) and 19.6% (95% CI 14.7 – 25.2%) for CIDR1 $\alpha$ , DBL2 $\beta$  and DBL5 $\beta$  respectively ( $p < 0.0001$  for comparison of overall recognition of DBL1 $\alpha$  or DBL4 $\gamma$  with any other domain Wilcoxon ranksum).



**Figure 5.1**

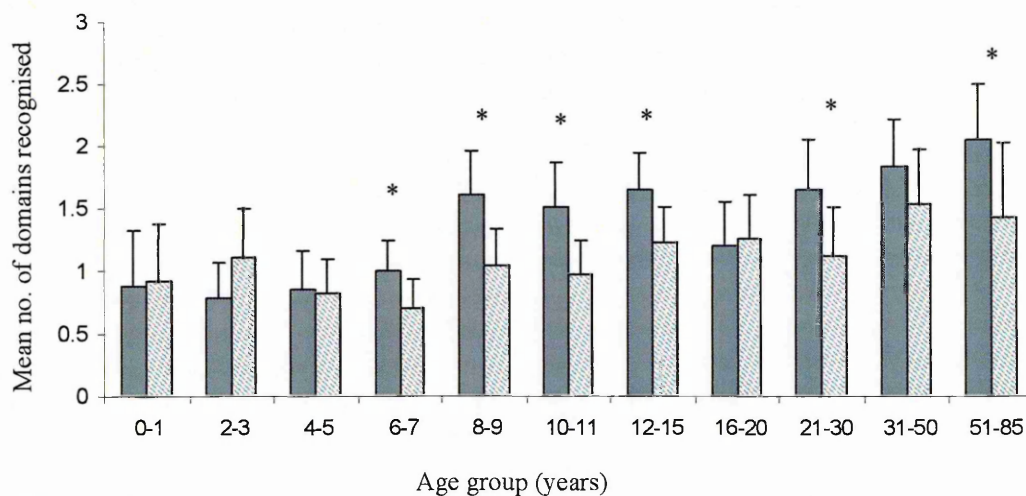
### Proportion of individuals in each age group recognising recombinant domains

Sera from individuals older than one year of age, were tested for reactivity against each recombinant protein in turn using ELISA. All individuals were tested in duplicate and if a disparity between the results of greater than 15% was obtained the serum was retested. Responses were scored as positive if the average OD obtained was greater than the average plus 3 standard deviations of a panel of 20 non-malaria exposed donors. The solid grey bars refer to individuals resident in Chonyi and the hatched bars to individuals resident in Ngerenya. a) DBL1 $\alpha$ , b) CIDR1 $\alpha$ , c) DBL2 $\beta$ , d) DBL4 $\gamma$ , e) DBL5 $\beta$ . P-values given are chi-squared for trend, C = Chonyi N = Ngerenya

There was a clear increase in proportion of responders and rate of acquisition of responses in Chonyi compared to Ngerenya. For example with DBL4 $\gamma$  the maximum response in Chonyi occurs in the 16 – 20 year age group with 61.8% (95% CI 43.6 – 77.8%) of individuals in this age group recognising this domain. This is compared to the maximum response in Ngerenya against the same domain, which did not occur until the 51 – 85 year age group and was lower at 56.5% (95% CI 34.5 – 76.8%). Acquisition of antibody responses against DBL4 $\gamma$  was more rapid in Chonyi where, by the age of 8 - 9 years 45.2% (95% CI 32.5 – 58.3%) of children in Chonyi responding compared to 18.6% (95% CI 9.70 – 30.9%) of children resident in Ngerenya ( $p=0.0019$  Wilcoxon ranksum). For responses against DBL1 $\alpha$ , the situation was different with acquisition in both areas continuing throughout adulthood. The maximum response in both areas did not occur until the 51 – 85 year age group although as with DBL4 $\gamma$ , the maximum response was greater in Chonyi, 67.6% (95% CI 50.2 – 81.9%) than in Ngerenya at 43.5% (95% CI 23.2 – 65.5%) although this difference was not significant. Overall a greater proportion of individuals resident in Chonyi showed evidence of reactivity against DBL1 $\alpha$  and DBL4 $\gamma$  but not CIDR1 $\alpha$ , DBL2 $\beta$  or DBL5 $\beta$ . The two-sample Wilcoxon rank sum for the differences in response between the two locations gave the following significance values: DBL1 $\alpha$   $p=0.0128$ , CIDR1 $\alpha$   $p=0.0594$ , DBL2 $\beta$   $p=0.0774$ , DBL4 $\gamma$   $p=0.0000$  and DBL5 $\beta$   $p=0.3848$ . However, as previously described, individuals resident in Chonyi had a higher parasite rate than those in Ngerenya at the time of cross-sectional bleed, 41.2% (95% CI 35.5 – 46.8%) of those under 10 years in Chonyi were parasitaemic at the time of bleed compared to 22.7% (18.0 – 27.4%) in Ngerenya ( $p<0.0001$ ) (figure 2.2). As a subsequent section will illustrate,

the presence of an asymptomatic parasitaemia resulted in a significantly greater likelihood of an individual recognising any domain compared to no domains.

Overall the number of domains recognised increased with age in both areas (Figure 5.2). In six of the age groups, individuals resident in Chonyi recognised a significantly greater number of domains. The proportion of individuals not recognising any domains was significantly greater in Ngerenya compared to Chonyi (figure 5.3). This remained significant when stratifying for parasite status (data not shown). There was no difference in the number of domains recognised when individuals were analysed by  $\alpha$ -thalassaemia genotype, (wild-type, heterozygote or homozygote) or by sickle cell genotype, (sickle cell trait, HbAS, or normal, HbAA) (figure 5.4). Haemoglobin genotype also made no difference to the recognition of each individual domain, (data not shown), nor did limiting the analysis to only those domains showing age-associated acquisition, DBL1 $\alpha$ , DBL4 $\gamma$  and DBL5 $\beta$ .

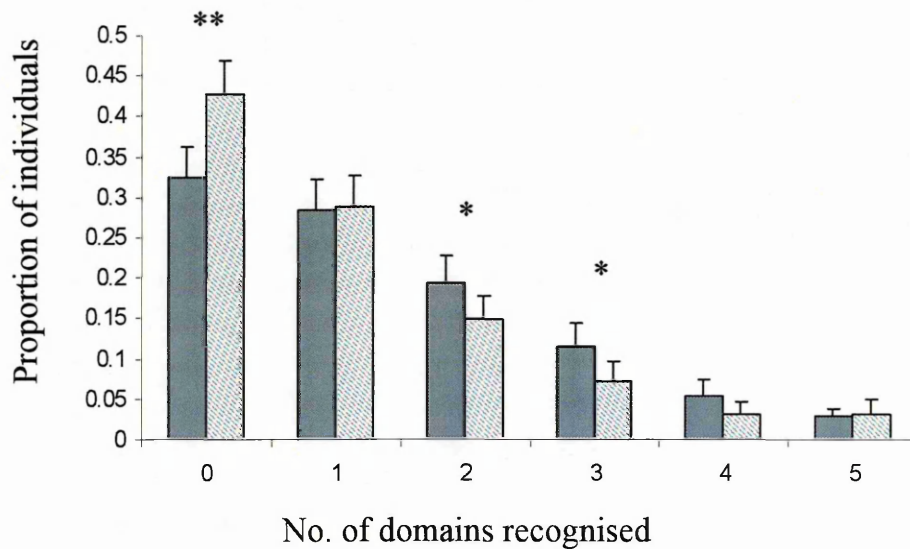


**Figure 5.2**

### Mean number of domains recognised by age

Graph shows mean number of domains, plus upper 95% confidence limits, recognised by individuals in each age group. The dark grey bars refer to individuals in Chonyi and the hatched bars to individuals resident in Ngerenya.

\* p value < 0.05



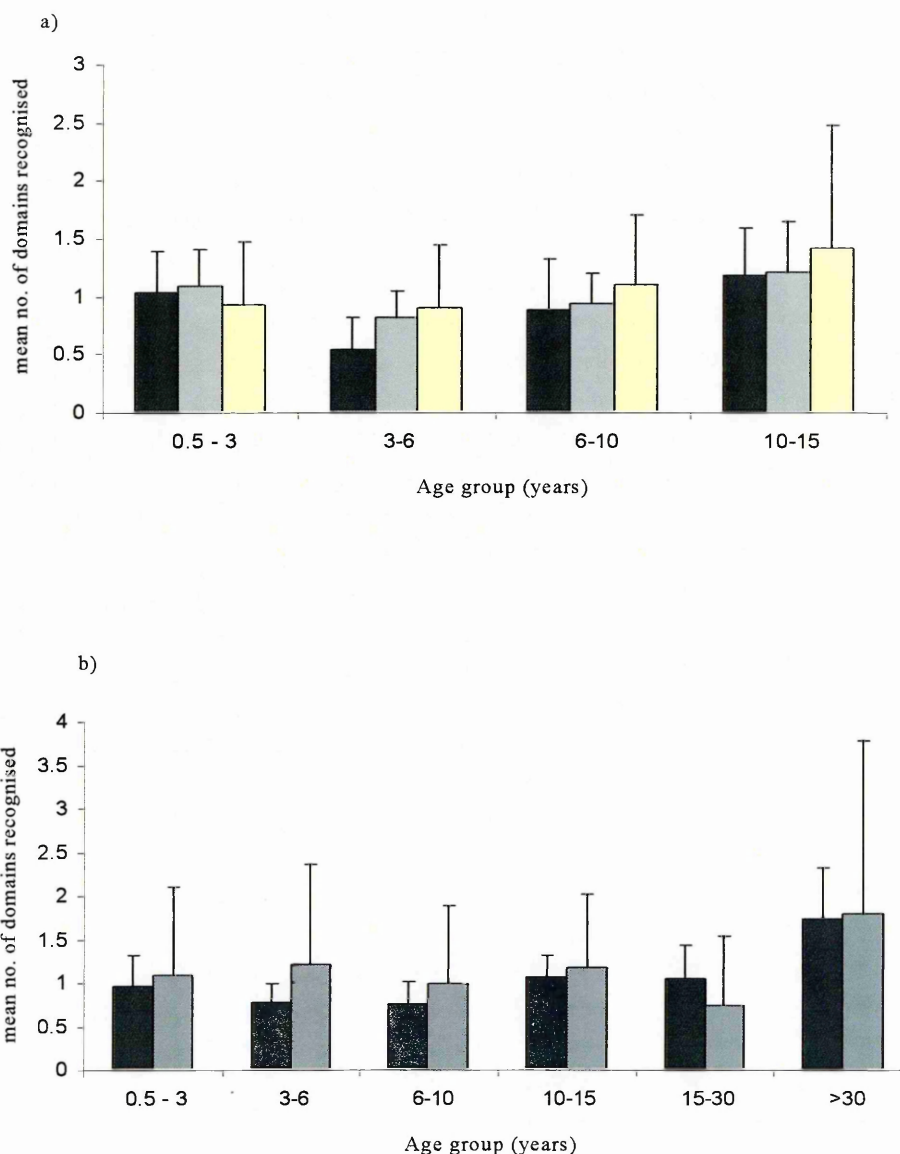
**Figure 5.3**

**Mean number of domains recognised by area**

Graph showing the proportion individuals resident in each area recognising either no domains, 1 2 3 4 or 5 domains. Shown are means plus upper 95% confidence limits. The dark grey bars refer to those individuals resident in Chonyi whereas the hatched bars refer to individuals resident in Ngerenya.

\* p value <0.05

\* \* p value <0.005



**Figure 5.4**

**a)  $\alpha$  Thalassaemia**

Dark grey bars refer to wild-type individuals, the light grey bars to heterozygotes for  $\alpha$  Thalassaemia and the cream bars are those individuals who are homozygote. Shown is the mean number of domains recognised in each age group with upper 95% confidence limits.

**b) Sickle cell trait**

Dark grey bars refer to wild-type individuals (AA genotype) and light grey bars those with sickle cell trait (AS genotype). Again, shown is the mean number of domains recognised in each age group with upper 95% confidence limits.

*Association of asymptomatic parasitaemia and anti-A4PfEMP1 antibody responses in Chonyi and Ngerenya*

It was noted in a previous study conducted in this area that analysed antibody reactivity to variant antigens exposed on the surface of infected erythrocytes, that the presence of asymptomatic parasitaemia at the time of serum collection resulted in a higher prevalence of antibodies, as measured by agglutination (Bull, Lowe et al. 2002). In this study marked variability in the relationship between the presence of parasites and reactivity of individual sera against each domain was seen. Using a logistic regression analysis taking into account age, exposure (as estimated by responses to whole schizont extract) and location, and restricting the analysis to those less than 10 years, individuals with parasites in their blood at the time of bleed were significantly more likely to recognise any recombinant domain compared to recognising no domains (OR 1.94 (95% CI 1.34 – 2.83)  $p < 0.001$ ). Interestingly this effect of parasitaemia on the presence of domain-specific antibodies was absent in those aged over 10 years in whom the presence of asymptomatic parasitaemia had no effect on the likelihood of recognising any recombinant domain (OR 1.22 (95% CI 0.81 – 1.83)  $p = 0.334$ ).

Using a multiple linear regression model, the effect of asymptomatic parasitaemia on the OD value obtained for each domain after controlling for age, location and exposure was determined. Having detectable parasites at cross-sectional bleed was associated with significantly higher OD values for domains DBL1 $\alpha$ , DBL2 $\beta$  and CIDR1 $\alpha$  (table 5.2). There was no effect on antibody reactivity against DBL4 $\gamma$  or DBL5 $\beta$ . Figure 5.5 shows graphically the odds ratios of recognising each domain if



an individual was parasite positive. Using a logistic regression model, again controlled for age, location and exposure, it was clear that the odds of recognising DBL1 $\alpha$  and DBL2 $\beta$  were significantly increased in the presence of detectable parasites.

*Individual variation in antibody responses to recombinant domains of A4-PfEMP1*

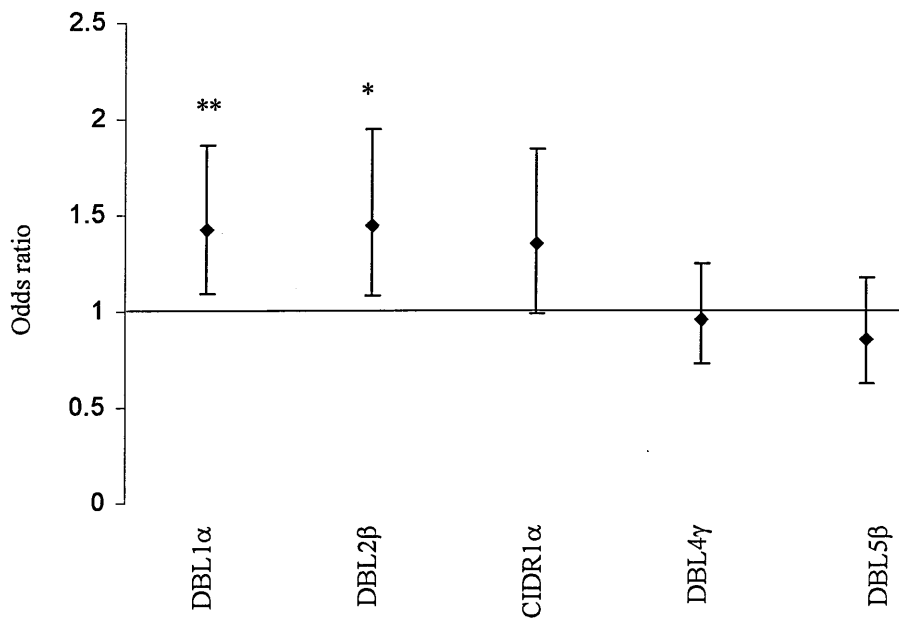
Extensive variation between individuals was found with regards to specificity of antibody response (Figures 5.6 and 5.7). Shown are examples of individuals from both areas with their antibody responses, measured as OD, graded into quartiles. As is obvious, there is marked variability between responses with some individuals (C719, C514, C672, N0847) showing high recognition of all five domains and others (C659, C754, N0546, N0007) showing poor responses to all domains. It was also clear that there were many individuals with responses directed at one domain over the others (C334, C712, N0862, N0518, N0456). 461 individuals recognised no domains and of these 57.7% were resident in Ngerenya and 42.3% in Chonyi. Of these, 61.2% were aged less than 10yrs and 110 (24.0%) were parasitaemic at the time of sampling. 350 individuals recognised 1 domain to the exclusion of all the others. 38 individuals recognised all 5 domains tested (3.11% of the whole cohort), of these 24 were aged over 10 yrs and interestingly of the 14 aged less than 10 years, 64.3% were aparasitaemic at the time of bleed.

**Table 5.2**

**Effect of parasite status on antibody levels to each recombinant domain (1)**

<b>Domain</b>	<b>coefficient<sup>1</sup></b>	<b>95% C.I.</b>	<b>p-value</b>
DBL1 $\alpha$	0.067	0.027 - 0.117	0.001
CiDR1 $\alpha$	0.044	0.015 - 0.072	0.003
DBL2 $\beta$	0.074	0.029 - 0.118	0.001
DBL4 $\gamma$	-0.036	-0.078 - 0.006	0.1
DBL5 $\beta$	0.002	-0.034 - 0.039	0.886

<sup>1</sup>Results from a multiple linear regression giving the coefficients for the effect of being parasite positive on the OD obtained to each domain, compared to having no parasites. Coefficients are controlled for the effects on age, location and exposure as estimated by responses to schizont extract.

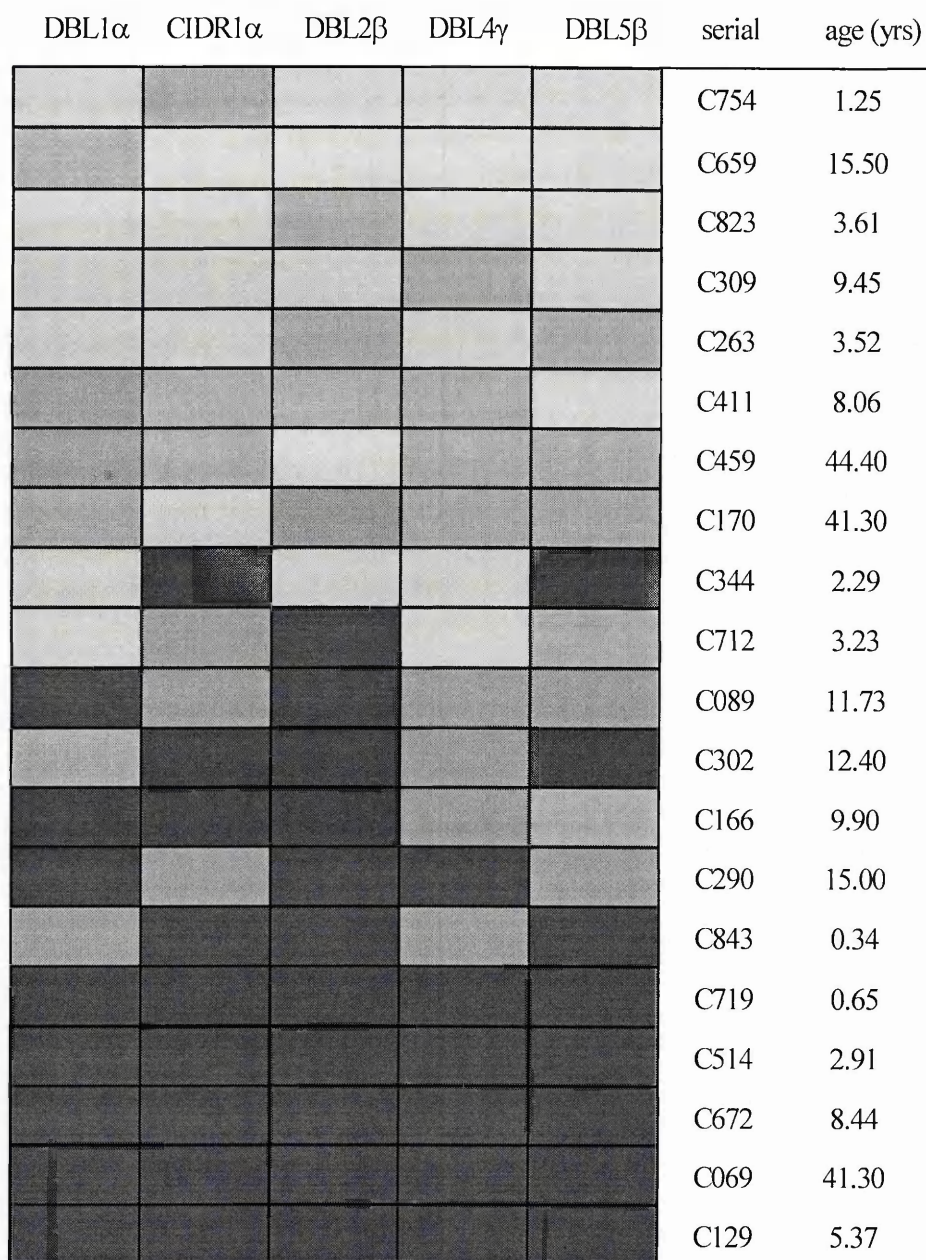


**Figure 5.5**

**Effect of parasite status on antibody levels to each recombinant domain (2)**

Graphical representation of the odds of being antibody positive for each domain if parasite positive at the time of cross-sectional bleed. The multiple logistic regression model used controlled for both age and location as possible confounders. Shown are the odds ratios with 95% confidence intervals. The solid line denotes an odds ratio of 1.

\* =  $p < 0.05$  \*\* =  $p < 0.005$

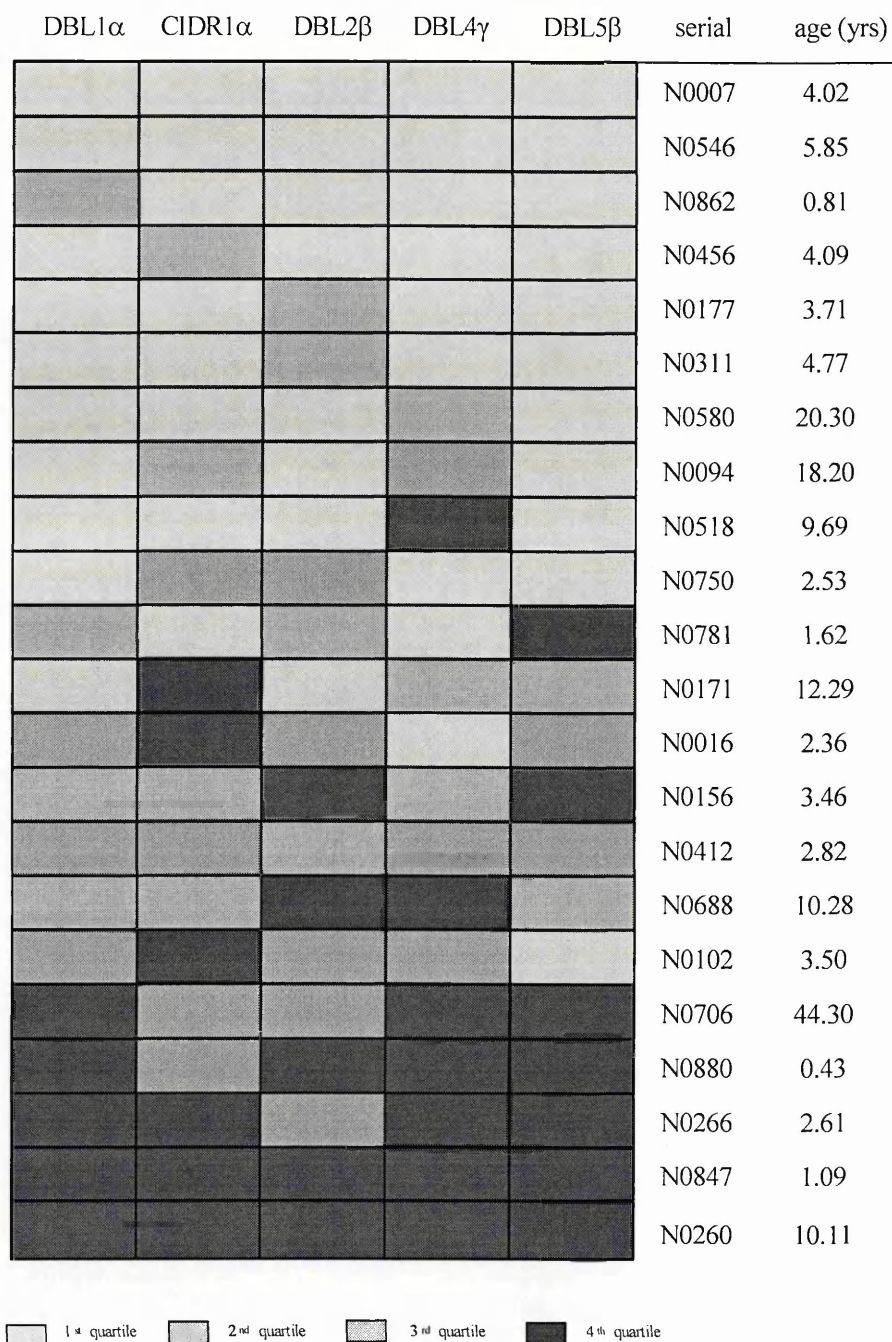


Key: 1<sup>st</sup> quartile 2<sup>nd</sup> quartile 3<sup>rd</sup> quartile 4<sup>th</sup> quartile

**Figure 5.6**

**Checkerboard illustrating a selection of individuals from Chonyi.**

The key refers to the intensity of reactivity obtained in ELISA to each domain presented along the top of each checkerboard, with the darkest blocks being OD's within the top 25% of responses and the lightest blocks being the lowest OD's obtained. Along the side of each checkerboard is the unique number given to each individual and their age in years.



**Figure 5.7**

**Checkerboard illustrating a representative selection of individuals from Ngerenya.**

The key refers to the intensity of reactivity obtained in ELISA to each domain presented along the top of each checkerboard, with the darkest blocks being OD's within the top 25% of responses and the lightest blocks being the lowest OD's obtained. Along the side of each checkerboard is the unique number given to each individual and their age in years.

There was evidence of a correlation between individual responses to each domain (table 5.3). A positive correlation existed between responses to all domains. To exclude this correlation simply representing cumulative exposure with age and transmission intensity a multiple linear regression was performed looking at the association between responses adjusted for age, location, parasite status and exposure as measured as responses to schizont extract as before. All positive correlations were maintained.

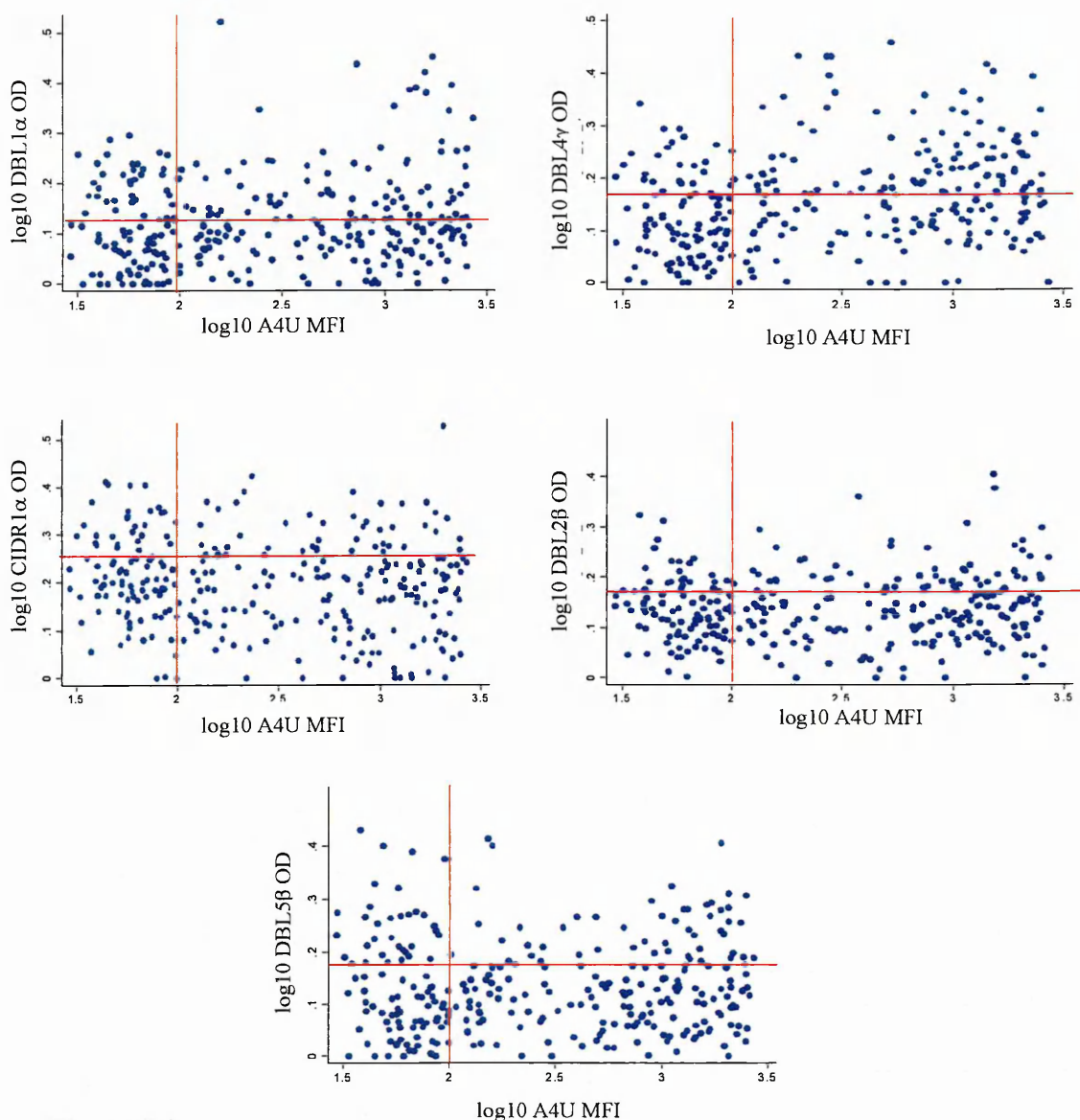
*Correlation of anti-domain antibody responses with antibody responses to the intact A4 parasitised erythrocyte*

Figure 5.8 shows scatter diagrams correlating responses to each domain against responses to intact A4U as measured by flow cytometry. Also shown are the cut off levels for positivity (the average of 20 non-exposed donors plus 3 standard deviations for each recombinant domain and the average of 20 non-exposed donors plus 3 standard deviations for responses against a4U) in red. Using Spearman's rank correlation coefficient to assess significance, only responses to DBL1 $\alpha$  and DBL4 $\gamma$  were positively correlated with responses against the intact parasitised erythrocyte, (table 5.4). Interestingly responses to CIDR1 $\alpha$  were significantly negatively correlated with responses to the surface of the intact erythrocyte. In an attempt to control for exposure accounting for any positive correlation, a multiple linear regression model was performed with age, location, parasite status and exposure (estimated by responses to schizont extract) as independent variables. Using this model, only responses to DBL4 $\gamma$  reached significance (data not shown).



**Table 5.3****Inter-domain correlations**

correlation	spearman's rho	p-value
DBL1 $\alpha$ DBL2 $\beta$	0.4368	<0.0001
DBL1 $\alpha$ CIDR1 $\alpha$	0.3738	<0.0001
DBL1 $\alpha$ DBL4 $\gamma$	0.3074	<0.0001
DBL1 $\alpha$ DBL5 $\beta$	0.4138	<0.0001
DBL2 $\beta$ CIDR1 $\alpha$	0.4269	<0.0001
DBL2 $\beta$ DBL4 $\gamma$	0.3123	<0.0001
DBL2 $\beta$ DBL5 $\beta$	0.4881	<0.0001
CIDR1 $\alpha$ DBL4 $\gamma$	0.1696	<0.0001
CIDR1a DBL5 $\beta$	0.3602	<0.0001
DBL4 $\gamma$ DBL5 $\beta$	0.3484	<0.0001



**Figure 5.8**

### **Correlations between responses to each recombinant domain and responses to A4U parasitised erythrocytes (1)**

Each scattergram shows correlation of individual responses, measured by ELISA, to each domain (y-axis) with responses to the intact A4- parasitised erythrocyte, measured by flow cytometry (x-axis). The red lines demonstrate the cut-off for positivity, (mean of responses from 20 non-exposed donors plus 3 standard deviations for the recombinant domains and mean of responses from 20 non-exposed donors plus three standard deviations for responses to the A4-parasitised erythrocyte).



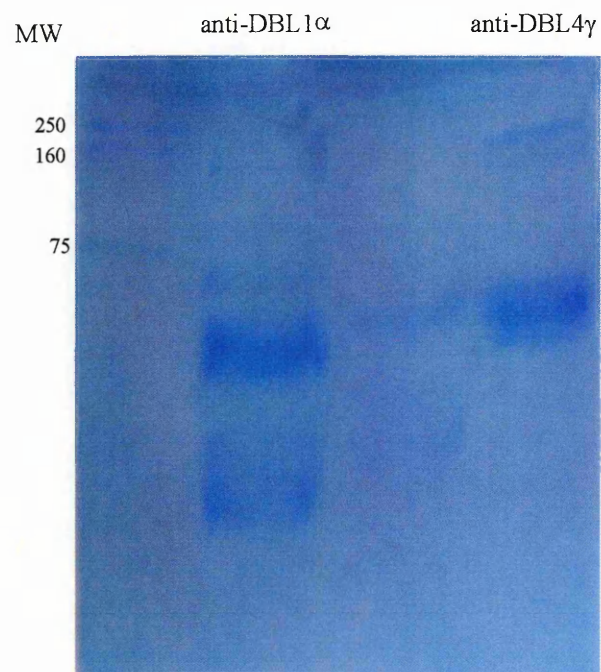
**Table 5.4**

**Correlations between responses to each recombinant domain and responses to A4U parasitised erythrocytes (2)**

correlation	spearman's rho	p-value
DBL1 $\alpha$ A4 Ultra	0.1256	<b>0.0324</b>
DBL2 $\beta$ A4 Ultra	0.0492	0.4038
CIDR1 $\alpha$ A4 Ultra	-0.1347	<b>0.0217</b>
DBL4 $\gamma$ A4 Ultra	0.2869	<b>0.0000</b>
DBL5 $\beta$ A4 Ultra	0.0408	0.4887

### *Affinity purification of anti-DBL1 $\alpha$ and anti-DBL4 $\gamma$ antibodies*

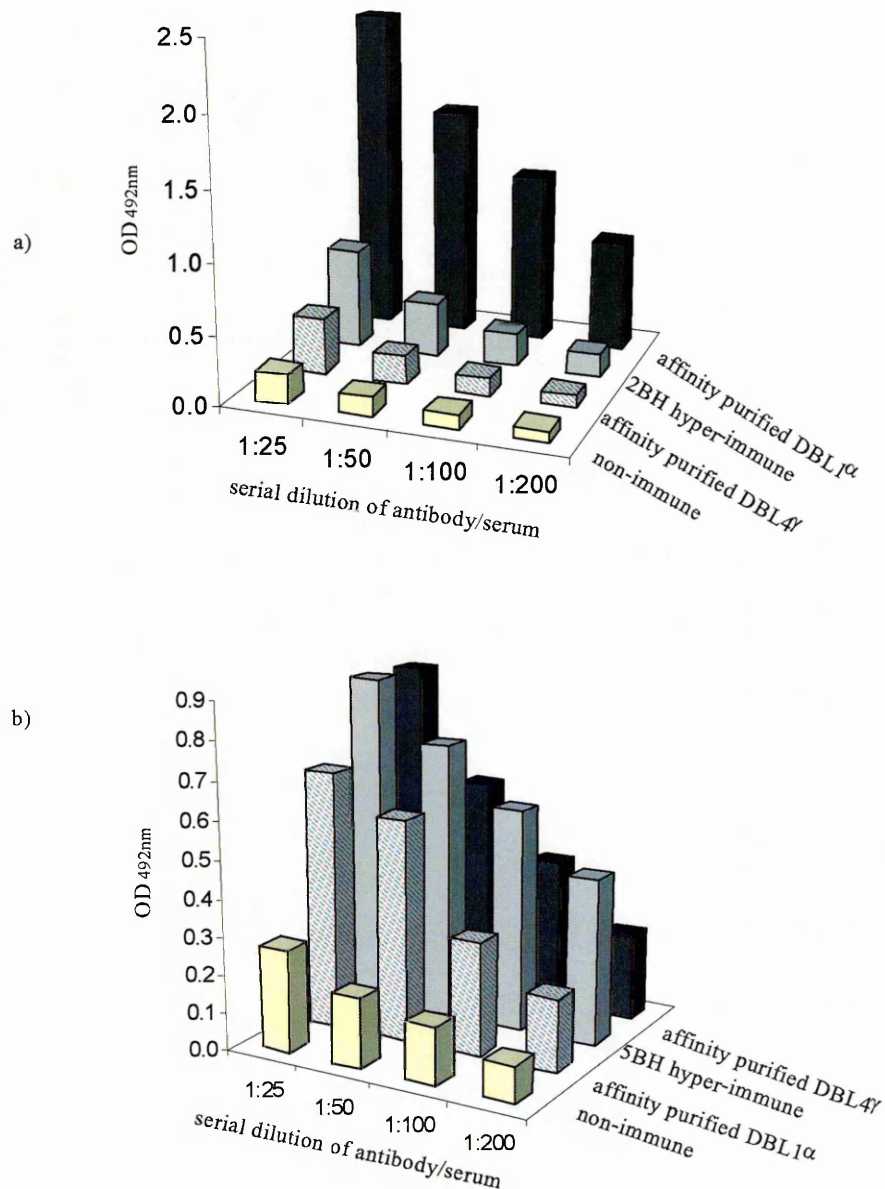
Following the purification and concentration process, (figure 5.9 shows SDS-PAGE gel of purified anti-DBL1 $\alpha$  and anti-DBL4 $\gamma$  antibodies) antibodies obtained demonstrated specificity of response against both purified DBL1 $\alpha$  and purified DBL4 $\gamma$  recombinant proteins (figure 5.10). However when responses were measured against both intact A4 parasitised erythrocytes and wild isolates obtained from symptomatic children by flow cytometry, no reactivity was observed. Experiments were repeated at varying dilutions of purified antibody to no avail. Whether these antibodies are reacting to epitopes exposed by the process of cloning and expression within the bacterial system, epitopes not exposed as part of the full A4 PfEMP1 molecule, or whether this reflects methodological limitations within the affinity purification process is presently unknown.



**Figure 5.9**

**SDS-PAGE gel**

Reducing gel showing the presence of 2 discrete bands corresponding to ~150KDa obtained after purification of sera on DBL1α and DBL4γ recombinant proteins.



**Figure 5.10**

### **ELISA specificity of affinity purified anti-DBL1α and anti-DBL4γ**

- graph of serial dilutions of antibody, both anti-DBL1α and anti-DBL4γ against purified recombinant DBL1α. Also shown are responses from a pool of 10 non-exposed donors and the previously screened highly positive sera from which the antibodies were purified (2BH)
- graph of serial dilutions of antibody, both anti-DBL1α and anti-DBL4γ against purified recombinant DBL4γ. Also shown are responses from a pool of 10 non-exposed donors and the previously screened highly positive sera from which the antibodies were purified (5BH)

## Discussion

PfEMP1 is currently the most plausible and best characterised of the erythrocyte surface-expressed parasite-induced proteins proposed as targets for the naturally acquired immune response (Bull and Marsh 2001). It has been hypothesised that naturally acquired immunity develops through the piecemeal acquisition of a large repertoire of antibodies directed against variants of this protein (Bull, Lowe et al. 1998). Supporting this possibility are vaccines based on selected domains from specific PfEMP1 variants which result in protection against the homologous genotype only (Baruch, Gamain et al. 2002).

PfEMP1 is a large, diverse and structurally complex molecule. It is composed of an intracellular, highly conserved domain and a large, extracellular variant domain. Although this extracellular domain is highly polymorphic between PfEMP1 variants, they share an overall common structure (figure 1.6). Through sequence and structural differences PfEMP1 variants are associated with diverse serological and functional properties. Understanding the naturally occurring antibody response to the DBL and CIDR domains making up the extracellular component of a specific PfEMP1 protein is an important part of evaluating the usefulness of PfEMP1 as a vaccine candidate. In this chapter, through the use of recombinant protein technology, the naturally acquired antibody response to the extracellular domains of A4 PfEMP1 in the sera of individuals resident in two areas endemic for *P. falciparum* malaria but with different transmission characteristics have been evaluated.

It is clear that there are differences in the total acquisition of domain-specific antibodies and the time over which such responses are acquired both between areas and between domains. Responses directed against CIDR1 $\alpha$  proved difficult to analyse satisfactorily. It was found that non-malaria exposed donors recognised this domain to high level, (figure 5.8), and so scoring individuals as antibody positive using the cut-off as defined resulted in very few individuals scoring positive. However it is clear from figure 5.8 that OD's obtained against CIDR1 $\alpha$  were comparable to those obtained for each of the other domains although there is no discernible age-associated acquisition. What is also clear is the spread of values obtained by the non-malaria exposed donors against the recombinant CIDR1 $\alpha$  domain was greater than for any of the other domains. It is not clear why individuals never exposed to malaria should recognise this particular domain, although it has been shown that CIDR1 $\alpha$  does bind non-immune immunoglobulins (Ig) (Chen, Heddini et al. 2000). More recently this particular domain of PfEMP1 was identified as a polyclonal activator of B cells from non-exposed donors (Donati, Zhang et al. 2004). This activation was specific to CIDR1 $\alpha$  and occurred through binding of CIDR1 $\alpha$  to the Fab fragment of immunoglobulins, both IgG and IgM, exposed on the B cell surface. It was also shown to bind directly to various human Ig fragments, total non-immune Ig and Ig from different species. However not all studies show the same non-immune binding, a recent study looked at IgG levels to recombinant CIDR1 $\alpha$  amongst adults and children resident in Tanzania and demonstrated very little reactivity by the non-exposed donors although it was striking that as in the results presented here, there was no age-associated acquisition of CIDR1 $\alpha$ -specific responses (Jensen, Magistrado et al. 2004). The situation is clearer with regards the other domains. All show acquisition with age and

differences are clear in the epidemiology of responses between those resident in the high and low transmission areas.

Previous studies have described an increased prevalence of antibodies to blood-stage antigens amongst individuals with detectable parasites at the time of bleed (Bull, Lowe et al. 2002; Kinyanjui, Mwangi et al. 2004; Polley, Mwangi et al. 2004). A similar result was obtained in this study with regard to responses to DBL1 $\alpha$ , DBL2 $\beta$  and CIDR1 $\alpha$ , but not to DBL4 $\gamma$  or DBL5 $\beta$ . This is a curious result for a number of reasons. For those domains showing an increased prevalence of antibodies, (DBL1 $\alpha$ , DBL2 $\beta$  and CIDR1 $\alpha$ ), as these recombinant domains were cloned from a laboratory parasite line, A4, it implies either that young children possess a degree of cross-reactive PfEMP1-specific antibodies capable of recognising individual domains or that these domains are present perhaps within expressed var genes within the pool of wild isolates in this area. For those domains where the presence of parasites makes no difference to their recognition (DBL4 $\gamma$  and DBL5 $\beta$ ), why should this be the case? Do these domains possess less cross-reactive epitopes within them or are they less ubiquitously present in nature?

In a previous chapter it was described how individuals were significantly more likely to recognise A4U-infected erythrocytes if they were carriers of the sickle cell trait (HbAS) (figure 3.15). It was with these results in mind that any association between the presence or absence of sickle cell trait and antibody recognition of each domain was examined. As is shown in this chapter, there was no significant association demonstrated between host genotype and antibody reactivity to each domain. The

question thus remains, what exactly is the target for the increased prevalence of antibodies against the surface of A4U-infected erythrocytes amongst those with HbAS genotype? Although these results do not exclude the possibility that the antigenic target is A4 *PfEMP1*, problems of maintaining conformation may account for the lack of any association, it is notable that similar increased prevalence of antibodies amongst those with HbAS have been shown for different randomly selected wild isolates (Marsh, Otoo et al. 1989; Cabrera, Cot et al. 2005).

There have been few studies examining directly the presence of naturally occurring *PfEMP1* domain-specific antibodies within the sera of individuals resident in malaria endemic areas. Oguariri and colleagues compared the prevalence of antibodies in the sera of children and adults resident in an area hyperendemic for *P. falciparum* transmission, directed against recombinant DBL1 $\alpha$  domains from 9 different wild isolates. There was a strong correlation between the age of the patients and the reactivity against the recombinant DBL1 $\alpha$  domains. There was no stratification of responses according to the presence or absence of detectable parasitaemia (Oguariri, Borrmann et al. 2001). Another study described antibody responses, measured by ELISA, against three conserved peptides from a laboratory strain of *PfEMP1* (Staalso, Khalil et al. 1998). These peptides corresponded to the relatively conserved N-terminal sequence containing the binding site for CD36 and the C-terminal sequence making up the intracellular portion of the integral membrane protein. The reactivity against the peptides increased with age and was also significantly greater in those individuals with an asymptomatic infection compared to those with symptoms of acute malaria. This result led to the tentative conclusion that the presence of peptide-specific antibodies was associated with asymptomatic



parasitaemia and therefore protection. However it must be noted that in this study those asymptotically parasitised were significantly older than those with symptoms.

The possibility exists that the antibody responses measured in this set of experiments do not accurately relate to those produced *in vivo*. By expressing each domain as a recombinant fusion protein, it may be that important conformational epitopes will not be accessible to the sera tested or equally that what is being recognised by sera are not important targets within the host. In order to address this, initially individual responses were compared to responses to the intact expressed A4 *Pf*EMP1 molecule on the surface of the parasitised erythrocyte. With two of the domains under study, DBL1 $\alpha$  and DBL4 $\gamma$  a positive correlation was found although once all responses had been adjusted for exposure surrogates, age, parasite status, transmission area and responses to schizont extract only DBL4 $\gamma$  remained significantly correlated. It was hoped that by purifying DBL4 $\gamma$ -specific antibodies from human sera it may be possible to show recognition of the parasitised erythrocyte by flow cytometry. Although the purified anti-sera reacted specifically with the recombinant domain it was purified from, unfortunately, no reactivity to the intact erythrocyte was demonstrated. Of note previous work has shown that immunisation of rabbits with recombinant proteins domains of A4 *Pf*EMP1 resulted in the production of polyclonal antisera, specific to each domain. Of these antisera, some recognised the surface of the A4 infected erythrocyte through flow cytometry. These included anti-DBL4 $\gamma$  and anti-DBL5 $\beta$  but not anti-CIDR1 $\alpha$  or anti-DBL1 $\alpha$  (Fagan 1999).

# Chapter 6

## Heterologous antibody responses and protection from clinical malaria

### Introduction

Non-sterile immunity to *P. falciparum* malaria is acquired by individuals living in endemic areas. This immunity enables people to maintain *P. falciparum* infections without the associated morbidity and mortality experienced by non-immune individuals (Marsh and Snow 1997). Evidence from the passive transfer of antibodies from immune to non-immune individuals suggests this immunity is at least in part, antibody mediated (Cohen, Mc et al. 1961; Cohen, McGregor et al. 1961; Edozien, Gilles et al. 1962). Studies examining humoral responses against the erythrocyte-surface and their association with protection from clinical disease have yielded conflicting results. Following infection individuals develop antibodies specific to the infecting isolate (Bull, Lowe et al. 1998; Giha, Staalsoe et al. 2000; Chattopadhyay, Sharma et al. 2003). These antibodies conferred protection against subsequent infection with that isolate, clinical malaria being caused by isolates expressing variants corresponding to gaps in the antibody repertoire of the infected child (Bull, Lowe et al. 1998). What is unknown currently is how relatively cross-reactive responses, assessed using heterologous isolates, are involved in protection from disease. Antibody responses to some isolates but not others appear to be associated with protection (Marsh, Otoo et al. 1989) (Bull, Lowe et al. 1998; Bull,

Kortok et al. 2000; Giha, Staalsoe et al. 2000; Dodoo, Staalsoe et al. 2001). The antigenic targets involved in the development of protective immunity are presently unknown.

In this chapter, using a longitudinal study design, the relationship between antibodies to antigens on the surface of four parasite lines and protection from clinical malaria was examined. In addition any effect of the presence of asymptomatic parasitaemia on this relationship was assessed and the relationship between antibodies to individual domains of one expressed *var* gene, A4 *PfEMP1* and disease incidence was explored.

## **Aims**

- 1) To establish the association of asymptomatic parasitaemia at cross-sectional survey on subsequent incidence of clinical malaria within a cohort of individuals aged 6 months to 10 years resident under different transmission characteristics on the coast of Kenya.
- 2) To examine the relationship between asymptomatic parasitaemia and antibody responses to four parasite lines in the context of future malaria experience.
- 3) To examine the association between antibody responses to the surface of erythrocytes infected with four parasite lines and protection from clinical non-severe malaria.
- 4) To examine the influence of asymptomatic parasitaemia, present at the time of blood assay, on this association.
- 5) To examine the association between antibody responses to five recombinant extracellular protein domains of A4 *PfEMP1* with protection from clinical malaria.

## Methods

The individuals' sampled were as described in chapter 3. A cross-sectional survey was performed in October 2000, when malaria transmission was low. Blood samples were obtained from children by venepuncture and plasma was separated by centrifugation and stored at -20°C. The children were examined for fever (axillary temperature was taken by use of an electronic thermometer), and their parasitological status was determined by use of a Giemsa-stained thin malaria smear. Individuals were followed up with weekly active malaria case detection and continuous passive case detection. A case of malaria was defined as:

Individuals aged less than 1 year – fever  $> 37.5^{\circ}\text{C}$  plus any parasitaemia

Individuals aged over 1 year – fever  $> 37.5^{\circ}\text{C}$  plus parasitaemia  $> 2500/\mu\text{l}$  (Mwangi 2003)

Antibody responses to the intact parasitised erythrocyte were measured by flow cytometry as outlined in chapter 3. Parasite lines used were as previous, A4U, A4 40-cycle, 3D7 and a clinical isolate P1. Antibody responses to each recombinant A4 PfEMP1 domain were measured by ELISA as outlined in chapter 5. Detailed descriptions of the methods used are given in chapter 2. Antibody positivity was defined as an MFI or OD greater than the mean plus 3 standard deviations of a panel of 20 non-exposed donors from Oxford U.K. Parasite positivity was defined as any parasites detected by microscopy at the time of cross-sectional survey. All individuals involved were asymptomatic and afebrile at the time of blood sampling.

### *Statistical analysis*

All statistical analysis was performed using Stata8™ (StataCorp. Ca. USA). The relationship between parasite status and reactivity to the surface of erythrocytes infected with each isolate with future disease experience was assessed using logistic regression. The outcome in every case was positivity for recognition of each isolate as classified using the cut-off of the mean response from 20 non-exposed donors plus 3 standard deviations. Initially the data were explored for additional factors affecting the likelihood of recognising each isolate. In every case these were identified as age (in categories of 6 months duration), location of each individual (either Chonyi or Ngerenya) and previous exposure to malaria infection (estimated by responses to whole schizont extract). The likelihood of recognition of each isolate was then compared in those individuals parasite positive and those parasite negative at cross-sectional survey after individuals had been stratified by future disease experience.

When the relationship between antibody responses, the presence of microscopically detectable parasites and clinical malaria was investigated, a multiple logistic model was performed after identification of significant confounding variables. These were identified as age (categorised as a factor of six months duration), location (Chonyi or Ngerenya) and exposure (estimated from responses to whole schizont extract). The outcome variable was at least one episode of malaria as defined previously. Individuals were categorised according to whether or not they had detectable parasitaemia at cross-sectional survey and were positive or negative for recognition of each isolate. This variable was then analysed as the main explanatory variable in

a multiple logistic regression with each group compared to the group of children both parasite and antibody negative.

When investigating the likelihood of having a greater number of episodes of clinical malaria, an ordered logistic regression was used. Individuals were scored 0, 1, 2, 3 or 4 according to the number of episodes of malaria they suffered during the six month follow up, the resultant odds ratio obtained gives the likelihood of being in a higher numbered group (i.e. having 2 episodes compared to 1, 3 compared to 2 etc) depending on which structured antibody/parasite group the individual fell into. Again confounding variable age, location and exposure were included.

Where differences in two continuous variables were assessed, no assumptions of distribution were made and the Wilcoxon ranksum test was used to calculate significance. Where more than two variables were compared the Kruskal Wallis test was used.

Kaplan-Meier survival curves were plotted using time in days to an episode as previously defined.

## **Results**

### *The epidemiology of malaria disease in Ngerenya and Chonyi*

As described in chapter 2, a previous study undertaken in this area defined the optimal clinical definition of malaria (children <1 year – fever greater than 37.5°C and any parasitaemia: children > 1 year – fever greater than 37.5°C and parasitaemia > 2500 parasites/ µl) and from this demonstrated a higher incidence of

clinical malaria disease in the lower of the two transmission areas under study (IRR 0.66 (95% CI 0.61 – 0.72)  $p < 0.001$  (Mwangi, Ross et al. 2005). For the individuals studied in this work, the odds of experiencing a clinical attack of malaria in Chonyi in those aged 5 to 10 years was significantly lower than the risk in those aged less than 5 years (OR 0.22 (95% CI 0.11-0.41)  $p < 0.0001$ ). Whereas in Ngerenya the odds of clinical attack in the older aged children was not significantly different from the children aged less than 5 (OR 1.27 (95% CI 0.79-2.03)  $p = 0.322$ ). Only in those aged 10 years or more in Ngerenya, was there a significant reduction in the likelihood of individuals suffering from malaria (OR 0.127 (95% CI 0.07-0.24)  $p < 0.001$ ).

At the time of cross-sectional bleed in October 2000, 43.2% of individuals in Chonyi and 23.6% in Ngerenya had parasites detectable by microscopy (figure 2.2). When the proportion of individuals suffering at least one attack of clinical malaria during the follow-up period was plotted by age, stratified by parasite status at the time of cross-sectional bleed, it was striking that among the children under the age of 24 months in Chonyi, a significantly greater proportion of those parasitised suffered at least one attack of malaria compared to those with no detectable parasites. This was less obvious in Ngerenya within the youngest age group but those aged between 12 and 24 months demonstrated clearly the same pattern of susceptibility, (figure 6.1). To further assess whether or not asymptomatic parasitaemia at cross-sectional survey identified a group of individuals at more risk of suffering an episode of clinical malaria in the subsequent six months, we first explored the data for any possible confounding influences using logistic regression. We identified age (in six month categories), location (either Chonyi or Ngerenya) and previous exposure (using

response to whole schizont as a proxy measure) as significant associations. The odds of experiencing an episode of clinical malaria, as defined above, was then compared in those parasite positive and parasite negative with the listed confounding variables included in a multiple logistic regression. Overall the odds ratio for becoming a case of malaria in the six months following cross-sectional bleed if parasite positive compared to parasite negative was 1.93 (95% CI 1.38 – 2.70)  $p < 0.0001$ . To exclude the possibility that the initial clinical disease episode was as a result of the asymptomatic parasitaemia detected at cross sectional survey, we excluded the first 30 days of follow-up after the cross-sectional bleed. Despite this there remained a significant increased likelihood of becoming a case if parasite positive (OR 1.49 (95% CI 1.10 – 2.20)  $p = 0.040$ ).

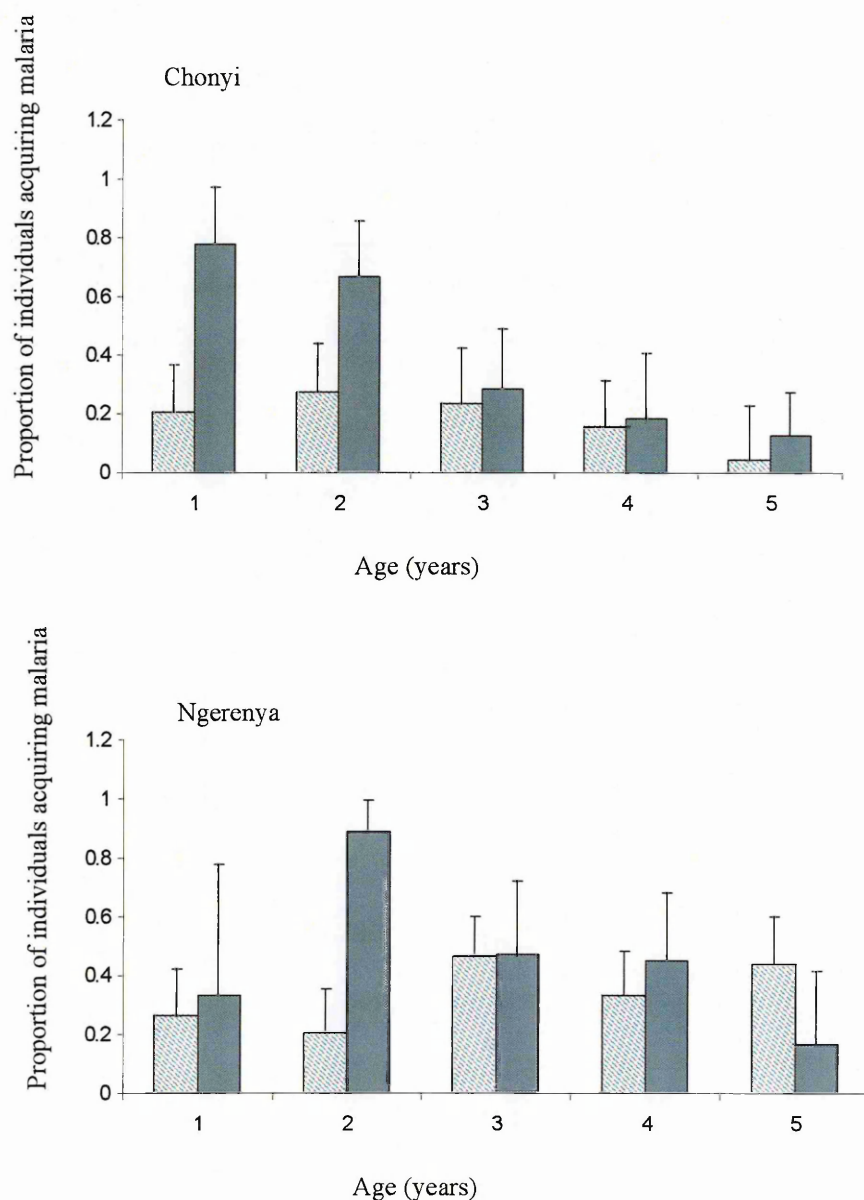
*The association between parasite status and antibody responses according to future disease experience*

As described in the preceding chapters, the presence of parasites at cross-sectional bleed was associated with significantly increased antibody responses to the intact parasite isolates A4, 3D7 and the wild isolate P1, also to the recombinant A4 PfEMP1 domains DBL1 $\alpha$ , DBL2 $\beta$  and CIDR1 $\alpha$ . However, if individuals were stratified according to whether or not they became a case of clinical malaria in the subsequent six months, then the relationship between the presence of parasites and antibodies to the infected erythrocyte surface was more complicated than at first thought. In order to establish whether this relationship was true whether or not individuals went on to become a case of clinical malaria, we initially checked for any variables other than parasite status, confounding the outcome of antibody positivity in a logistic regression. These were identified as age (in six month categories),



location, and exposure, as estimated by responses to whole schizont extract. A multiple logistic regression was then performed on individuals classified as either a control (no episode of malaria in the subsequent six months) or a case (at least one episode of malaria in the subsequent six months). The results are displayed in table 6.1. There is a marked difference in the relationship between antibody positivity and the presence of parasites between the cases and controls. There is no apparent association between recognising each of the isolates tested and the presence of asymptomatic parasitaemia in those who went on to become a case of malaria. In contrast there is a definite and clear positive association between the likelihood of recognising each individual isolate and the presence of parasites in those apparently protected from clinical malaria, at least in the subsequent six months.

We then went to do the same analysis on responses to individual A4 PfEMP1 domains (table 6.2). As already discussed, only three domains showed an increased likelihood of antibody positivity in the presence of parasites; DBL1 $\alpha$ , DBL2 $\beta$  and CIDR1 $\alpha$ . When this relationship was analysed by future disease experience as above, the results were less clear cut. Whereas responses to DBL1 $\alpha$  and DBL2 $\beta$  were increased in likelihood in the presence of parasites in those acting as controls, as was found for responses to the intact infected red cell, responses to CIDR1 $\alpha$ , DBL4 $\gamma$  or DBL5 $\beta$  were not similarly increased in this subgroup of individuals. It is notable that responses to DBL1 $\alpha$  were also increased in those parasitaemic who became a case and that responses to CIDR1 $\alpha$  showed the opposite association, an increased likelihood of response if parasitaemic in those who did become a case as opposed to those who were apparently protected.



**Figure 6.1**

### Average numbers of individuals suffering at least one episode of malaria

Shown are the proportion (plus upper 95% confidence limit) of individuals in each year group suffering at least one episode of malaria (as defined in the text) over the follow-up period in both areas. Individuals are stratified into those parasite positive (grey bars) and parasite negative (hatched bars) at the time of cross-sectional bleed.

**Table 6.1 Association between antibody responses and parasite positivity stratified by future disease experience**

Individuals with no episodes of malaria<sup>1</sup>

Isolate	Odds ratio <sup>2</sup>	95% confidence interval	p-value
A4U	6.18	2.15 - 17.7	0.001
A4 40-cycle	12.6	2.98 - 53.12	0.001
3D7	11.97	2.43 - 58.9	0.002
Clinical isolate P1	4.03	1.07 - 15.09	0.039

Individuals with at least one episode of malaria<sup>1</sup>

Isolate	Odds ratio <sup>2</sup>	95% confidence interval	p-value
A4U	1.7	0.43 - 6.71	0.44
A4 40-cycle	1.34	0.35 - 5.09	0.07
3D7	0.83	0.19 - 3.41	0.79
Clinical isolate P1	0.64	0.12 - 3.31	0.59

<sup>1</sup>Malaria defined as: individuals aged >1year - fever >37.5°C plus parasitaemia>2500/μl, individuals aged <1year - fever>37.5°C plus any parasitaemia

<sup>2</sup>Odds ratio refers to likelihood of being antibody positive, for responses against each isolate in turn, if parasite positive compared to parasite negative at cross-sectional survey. All individuals stratified initially by whether or not they experienced at least one episode of clinical malaria in the six months subsequent to the bleed.

*Association of antibodies to the intact infected-erythrocyte surface and protection from clinical malaria*

We next explored whether children who subsequently became a case of clinical malaria (cases) differed in their antibody responses to the parasite isolates tested, compared to children who did not become a case (protected). As the rate of acquisition of immunity differed between the two areas as described, individuals were examined over different age ranges in each area. In Chonyi differences in antibody levels were assessed in those individuals aged between 6 months and 5 years were assessed, with responses in Ngerenya examined over the age range 6 months to 10 years. Overall there were no significant differences over the age groups described between antibody reactivity to A4U in those who became future cases compared to those who did not ( $p=0.4292$  between individuals resident in Chonyi and  $p=0.1406$  between individuals resident in Ngerenya, Wilcoxon ranksum). No significant differences were found with responses to any other isolate tested (data not shown). However, if individuals were categorised according to whether or not they had a microscopically detectable parasitaemia at cross-sectional survey, then marked differences were apparent between those who became cases and those who did not, at least in Chonyi (figure 6.2). Amongst those children parasite positive at cross-sectional survey, there was a clear and significant difference between those classified as future cases and those apparently protected, with those children not suffering from

**Table 6.2 Association between anti-domain specific antibody responses and parasite positivity stratified by future disease experience**

Individuals with no episodes of malaria<sup>1</sup>

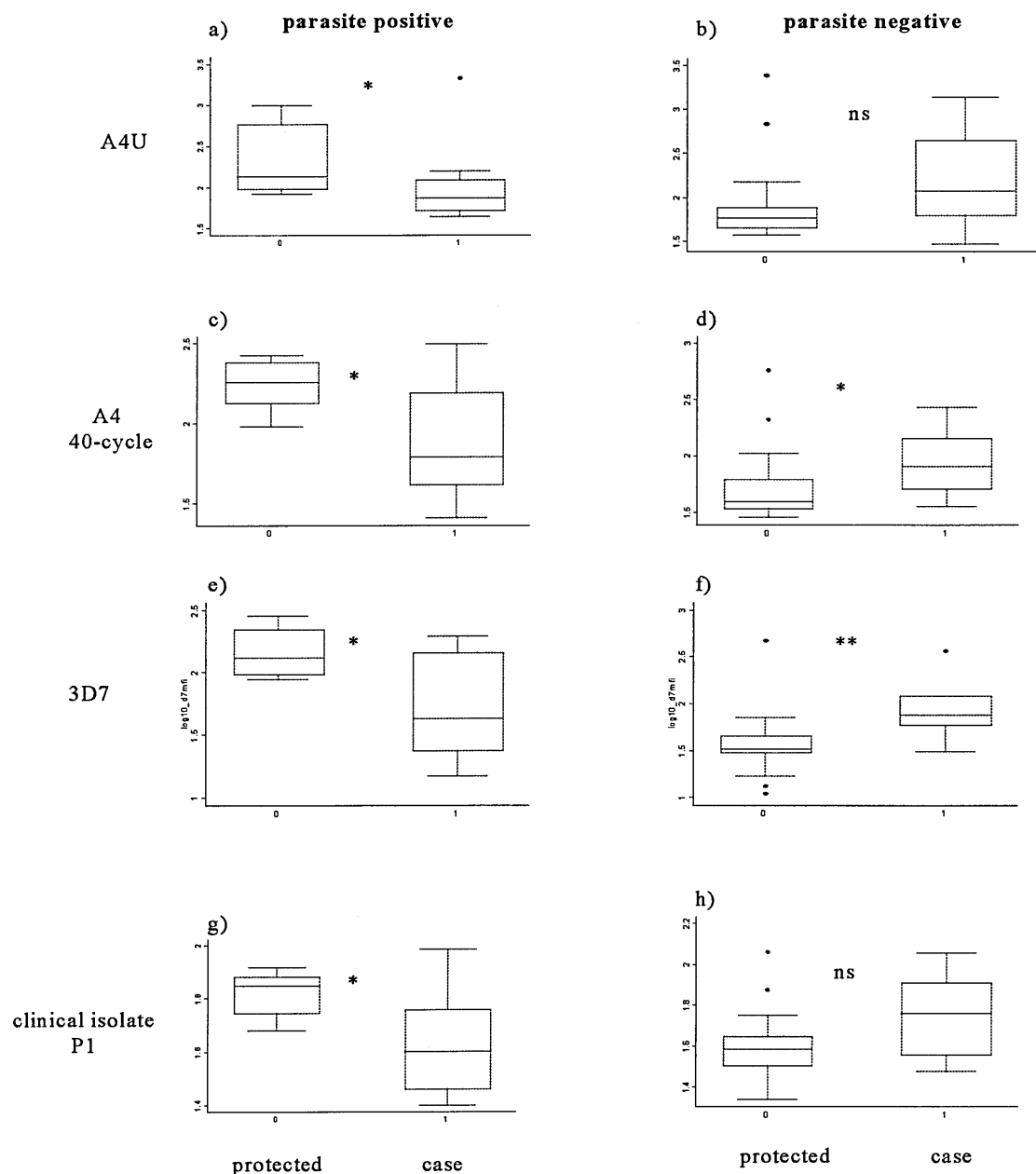
Domain	Odds ratio <sup>2</sup>	95% confidence interval	p-value
DBL1 $\alpha$	1.39	1.05 - 1.87	0.024
CIDR1 $\alpha$	1.52	1.10 - 2.10	0.01
DBL2 $\beta$	1.19	0.84 - 2.10	0.33
DBL4 $\gamma$	0.91	0.67 - 1.21	0.513
DBL5 $\beta$	0.76	0.53 - 1.08	0.125

Individuals with at least one episode of malaria<sup>1</sup>

Domain	Odds ratio <sup>2</sup>	95% confidence interval	p-value
DBL1 $\alpha$	1.99	1.00 - 3.95	0.049
CIDR1 $\alpha$	1.25	0.60 - 2.58	0.552
DBL2 $\beta$	2.96	1.42 - 6.16	0.004
DBL4 $\gamma$	1.45	0.69 - 3.06	0.325
DBL5 $\beta$	1.69	0.78 - 3.66	0.185

<sup>1</sup>Malaria defined as: individuals aged >1year - fever >37.5°C plus parasitaemia>2500/ $\mu$ l, individuals aged <1year - fever>37.5°C plus any parasitaemia

<sup>2</sup>Odds ratio refers to likelihood of being antibody positive, for responses against each domain in turn, if parasite positive compared to parasite negative at cross-sectional survey. All individuals stratified initially by whether or not they experienced at least one episode of clinical malaria in the six months subsequent to the bleed.



**Figure 6.2**

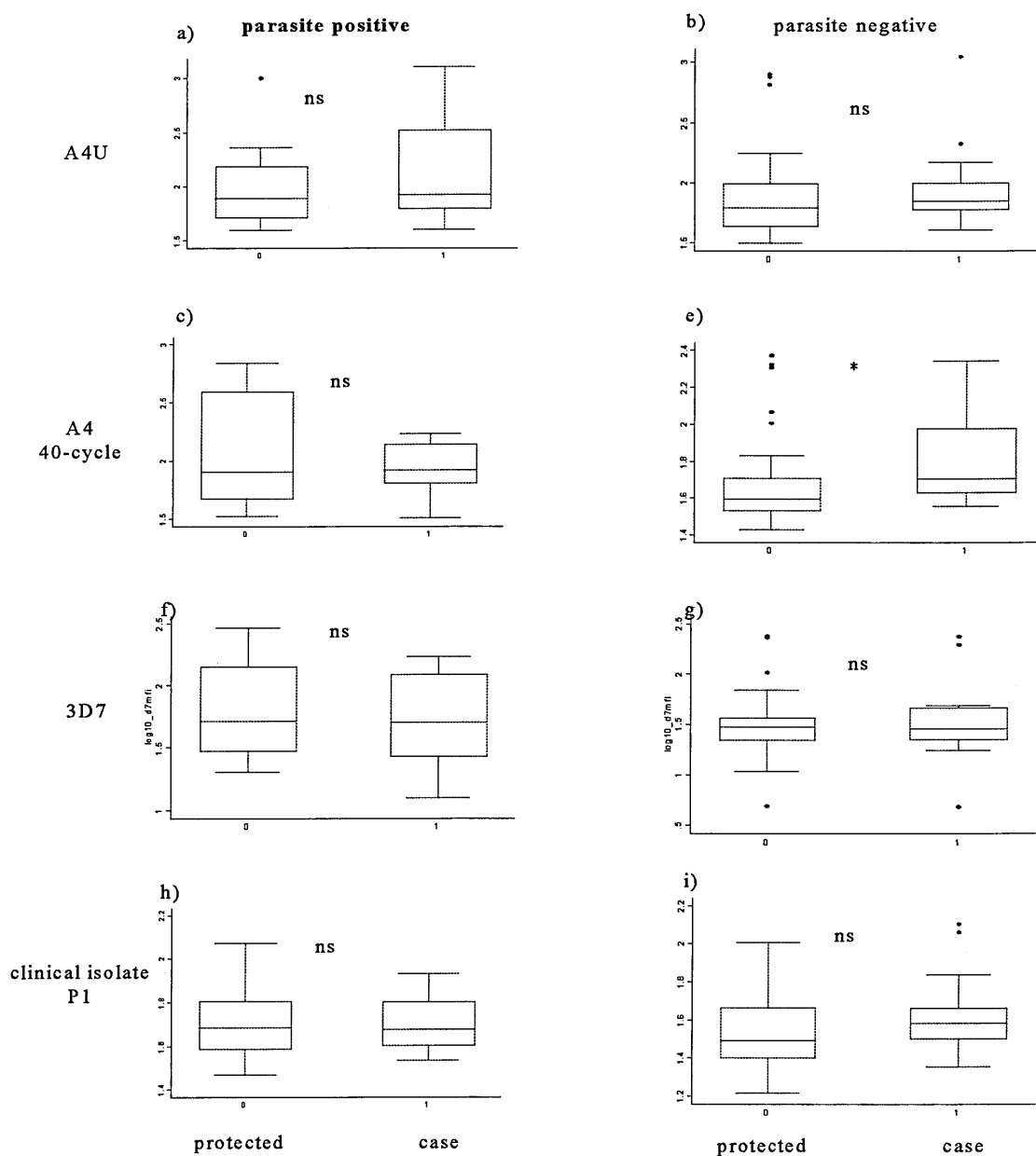
### Antibody responses according to parasite status and future disease experience

#### 1. Chonyi

Box and whisker plots showing median MFI (log transformed) plus interquartile range. Individuals are categorised according to whether they had microscopically detectable parasitaemia at cross-sectional bleed (graphs a, c, e and g) or not (graphs b, d, f and h) and also according to whether they experienced at least one case of clinical malaria (case) or not (protected). Responses to each parasite are shown.

\* p<0.05

\*\* p<0.005 ns not significant



**Figure 6.3**

**Antibody responses according to parasite status and future disease experience**

## 2. Ngerenya

Box and whisker plots showing median MFI (log transformed) plus interquartile range. Individuals are categorised according to whether they had microscopically detectable parasitaemia at cross-sectional bleed (graphs a, c, e and g) or not (graphs b, d, f and h) and also according to whether they experienced at least one case of clinical malaria (case) or not (protected). Responses to each parasite are shown.

\* p<0.05

an episode having significantly increased antibody levels measured against each of the isolates tested. In Ngerenya, no significant differences were seen in those individuals parasite positive, possibly because of a lower parasite prevalence in this area (figure 6.3). Those children with no microscopically detectable parasitaemia showed a markedly different pattern of responses (figures 6.2 and 6.3 - graphs b, d, f, h). In Chonyi, there was significantly less antibody present within individuals apparently protected, against the parasite lines A4 40-cycle and 3D7, and although there was no significant difference between responses to A4U and the clinical isolate P1, the trend was the same. In Ngerenya, again differences were on the whole not significant although the general trend was the same, the exception being antibody levels to A4 40-cycle which showed differences in the same direction as those seen in Chonyi.

The notable effect the presence of a microscopically detectable parasitaemia had on the association of antibody responses and clinical malaria was further evident in a Kaplan-Meier survival curve (figure 6.4). Although overall there was no significant difference in the cumulative proportion of individuals experiencing clinical malaria between those individuals scoring positive for antibody recognition of A4U and those scoring negative, when individuals were stratified by parasite status, then there was a significant survival advantage amongst those antibody positive and parasite positive.

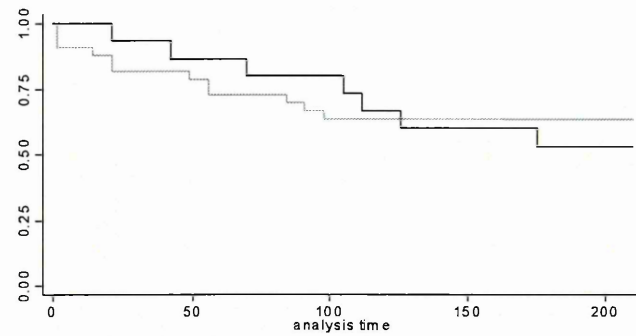
These results led us to categorise individuals into 4 groups depending on both their antibody status in response to each isolate (either positive or negative as defined



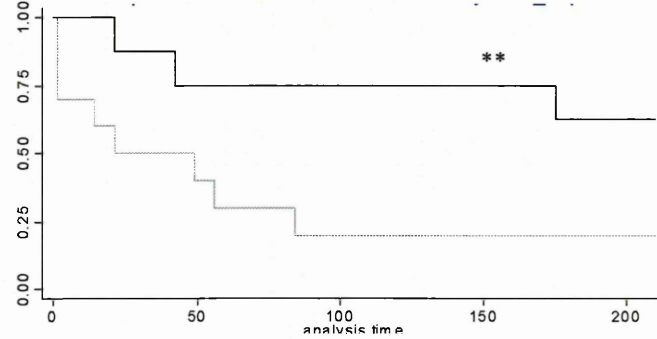
previously) and parasite status at cross-sectional survey (either microscopically detectable parasites present, i.e. parasite positive or absent, parasite negative). The four groups were as such:

Group	Antibody status	Parasite status
ab + pf +	positive	positive
ab + pf -	positive	negative
ab - pf +	negative	positive
ab - pf -	negative	negative

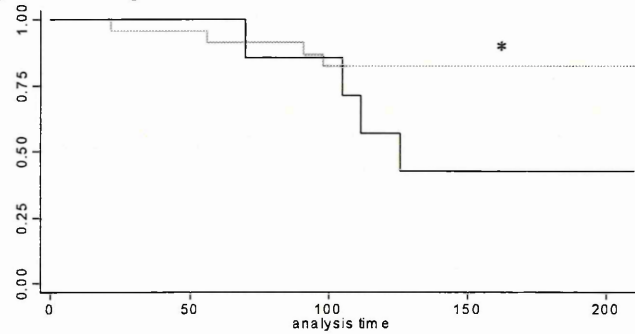
a) All individuals



b) Parasite positive individuals



c) Parasite negative individuals



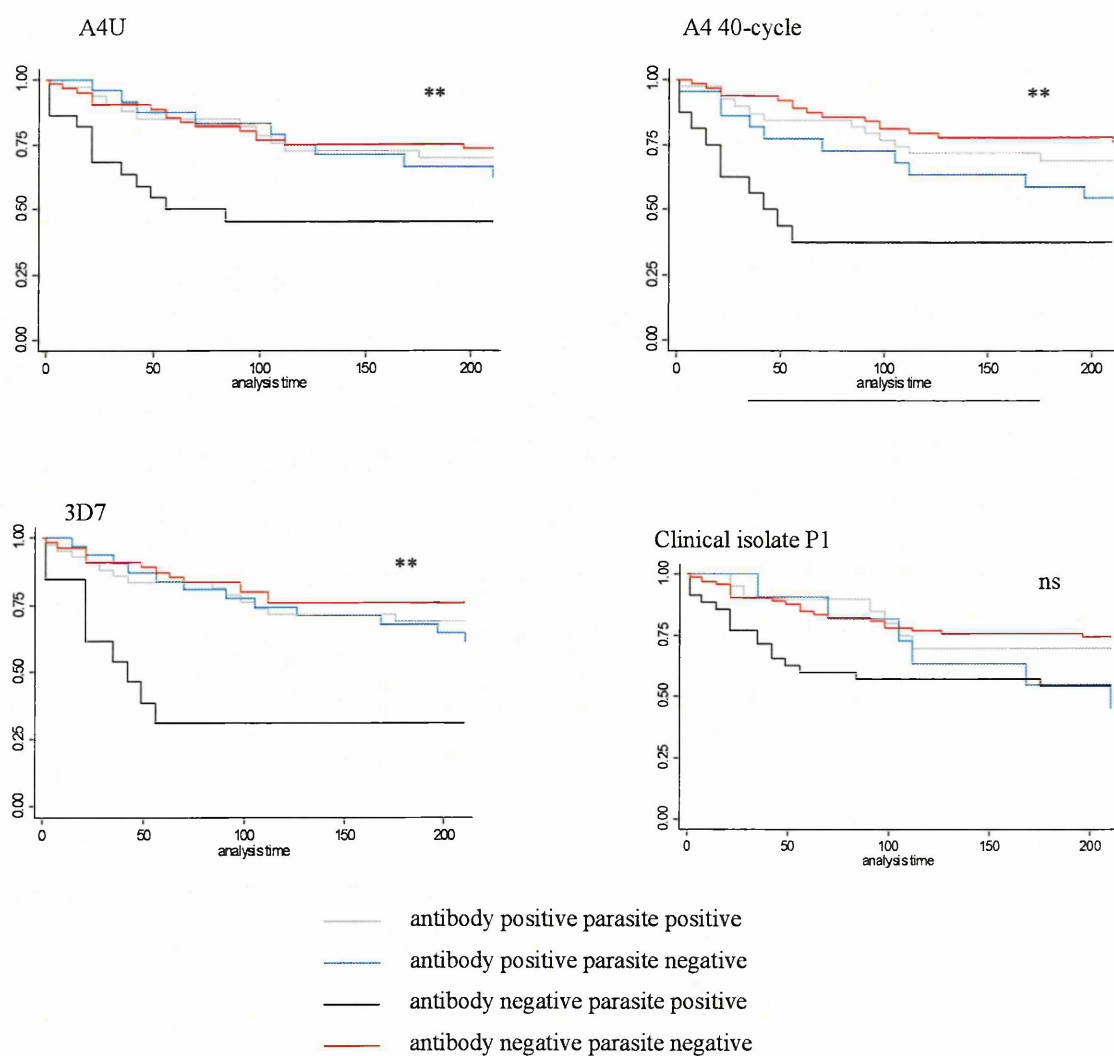
**Figure 6.4**

### **Kaplan-Meier survival curve according to antibody status.**

Graphs show proportion of individuals experiencing clinical malaria with time in days over the six month follow up period. Individuals are categorised according to whether they scored positive for recognition of A4U at cross-sectional bleed (black lines) or not (grey lines).

\*  $p < 0.05$  (logrank)

\*\*  $p < 0.005$  (logrank)

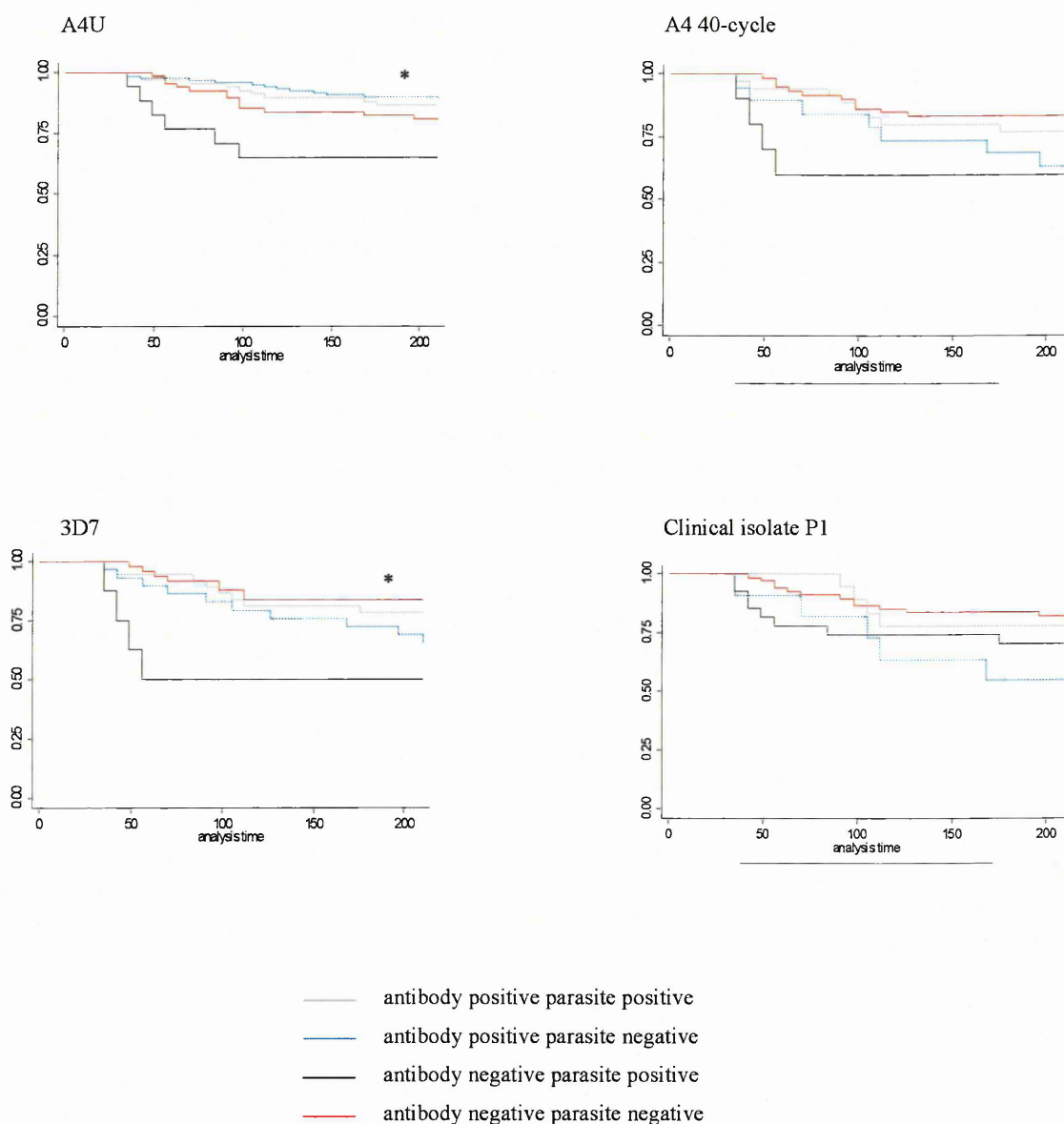


**Figure 6.5**

**Kaplan-Meier survival curve according to antibody and parasite status.**

Graphs show proportion of individuals experiencing clinical malaria with time in days over the six month follow up period. Individuals are categorised according to whether they scored positive for recognition of each parasite line in turn and whether or not they had microscopically detectable parasites at cross-sectional bleed

\*\* $p < 0.005$  (logrank)



**Figure 6.6**

**Kaplan-Meier survival curve according to antibody and parasite status with first 30 days of follow up censored.**

Graphs show proportion of individuals experiencing clinical malaria with time in days over the six month follow up period. Individuals are categorised according to whether they scored positive for recognition of each parasite line in turn and whether or not they had microscopically detectable parasites at cross-sectional bleed

\*= $p < 0.05$

Figure 6.5 shows the Kaplan-Meier curves obtained for each of the four groups. As is obvious the group of children with parasites detected at cross-sectional survey but no concomitant heterologous antibody response to all parasite lines appeared to be differentially susceptible to clinical malaria. There remained a significant difference against two of the parasite lines tested if the first 30 days were censored (figure 6.6).

Examining the base-line characteristics of the children in each of the four groups revealed some important differences. Children both antibody positive and parasite positive with regards all four parasite lines, were significantly older than children in the other three groups (data not shown). There were no significant differences between the ages of the children in the other three groups. For this reason the likelihood of acquiring at least one episode of clinical malaria in the subsequent six months was investigated according to group in a multiple logistic model with age (categorised into six month intervals), location and exposure (estimated from responses to whole schizont extract) as confounding variables. Table 6.3 shows the results for responses to A4U overall and within each location separately. Both overall and within Chonyi only, those children parasite positive at cross-sectional survey with no concomitant antibody response to A4U had a significantly increased risk of experiencing at least one episode of clinical malaria in the subsequent six months. This remained so if the first 21 days after the cross-sectional survey was censored. The same pattern was observed with responses to the other three parasite lines overall (table 6.4).

When the outcome examined was number of malaria episodes, an ordered logistic regression model was used with the same confounding variables included. Again the group of children antibody negative and parasite positive were significantly more likely to experience a greater number of malaria episodes in the subsequent six months. This was true for all parasite lines tested (table 6.5).

*Association of anti-A4PfEMP1 domain-specific antibody responses and protection from clinical malaria in Chonyi and Ngerenya*

In order to investigate any association of domain-specific antibody responses with protection from clinical malaria, initially the univariate association between detectable serum IgG to each antigen (measured as OD), and whether or not the individual had an episode of clinical malaria during the follow-up period was investigated by fitting the continuous variable against a binomial distributed outcome using logistic regression. As antibody reactivity is known to increase with exposure, a multiple logistic regression was then performed with age, converted to a factor of age-groups of six months duration, location either Chonyi or Ngerenya and reactivity to schizont extract, as a marker of exposure, expressed as a continuous variable included as co-dependent variables. Results are expressed as odds ratios per 10 fold increase in antibody levels.

When the antibody positivity to the PfEMP1 recombinant domains were analysed as a whole within Chonyi, no association was found between antibody response and subsequent disease experience (table 6.6). However by categorising individuals into those who were parasite positive at the time of bleed and those parasite negative, a positive association between the presence of anti-DBL1 $\alpha$  antibodies and protection from subsequent clinical malaria was evident in those parasite negative at the time of

bleed. No association with protection from or susceptibility to malaria was observed with antibodies against any other recombinant domain in Chonyi and antibody responses to none of the domains were associated with protection in Ngerenya (table 6.7).

**Table 6.3 Association of anti-A4U antibodies and parasites with protection from clinical malaria**

**a) All individuals, both areas**

Group <sup>1</sup>	total number	No. with malaria <sup>2</sup> (%)	Odds ratio <sup>3</sup> (95% C.I.)	p-value
ab+ pf+	70	12 (17.14)	1.31 (0.52 – 3.37)	0.563
ab+ pf -	120	14 (11.67)	1.04 (0.41 – 2.71)	0.921
ab- pf+	24	13 (54.17)	3.20 (1.20 – 8.51)	0.020
ab- pf -	74	19 (25.68)	1	1

**b) Individuals resident in Chonyi**

Group <sup>1</sup>	total number	No. with malaria <sup>2</sup> (%)	Odds ratio <sup>3</sup> (95% C.I.)	p-value
ab+ pf+	43	7 (16.28)	4.08 (0.76 – 21.46)	0.097
ab+ pf -	61	6 (9.84)	5.50 (1.01 – 29.92)	0.048
ab- pf+	12	8 (66.67)	12.07 (2.24 – 64.91)	0.004
ab- pf -	31	4 (12.90)	1	1

**c) Individuals resident in Ngerenya**

Group <sup>1</sup>	total number	No. with malaria <sup>2</sup> (%)	Odds ratio <sup>3</sup> (95% C.I.)	p-value
ab+ pf+	27	5 (18.52)	0.70 (0.11 – 4.42)	0.709
ab+ pf -	59	8 (13.56)	0.65 (0.14 – 3.06)	0.588
ab- pf+	12	5 (41.67)	1.17 (0.30 – 4.68)	0.823
ab- pf -	43	15 (34.88)	1	1

<sup>1</sup>Individuals were stratified according to whether they were antibody positive (scored as corrected MFI greater than the mean plus 3 standard deviations of the MFI of 20 non-exposed donors) or negative and parasite positive or negative (detected by microscopy) at cross-sectional survey.

<sup>2</sup>Malaria defined as at least one episode of fever>37.5°C plus parasitaemia>2500/μl if aged older than one year and fever >37.5°C plus any parasitaemia if aged less than one year.

<sup>3</sup>Odds ratio of becoming a case of malaria obtained from a multiple logistic regression controlling for age (categorised as a factor of six months duration) and exposure (estimated by responses to whole schizont extract)



Table 6.4 Association of antibodies to three parasite lines and the presence of parasites with protection from clinical malaria

a) Association of anti-A4 40-cycle antibodies and parasites with protection from clinical malaria

Group <sup>1</sup>	total number	No. with malaria <sup>2</sup> (%)	Odds ratio <sup>3</sup> (95% C.I.)	p-value
ab+ pf+	39	12 (30.77)	1.55 (0.55 – 4.34)	0.402
ab+ pf -	22	10 (45.45)	2.88 (0.94 – 8.82)	0.064
ab- pf+	16	10 (62.5)	5.35 (1.66 – 17.18)	0.005
ab- pf -	63	15 (23.81)	1	1

b) Association of anti-3D7 antibodies and parasites with protection from clinical malaria

Group <sup>1</sup>	total number	No. with malaria <sup>2</sup> (%)	Odds ratio <sup>3</sup> (95% C.I.)	p-value
ab+ pf+	42	13 (30.95)	1.43 (0.53 – 3.91)	0.479
ab+ pf -	31	12 (38.71)	2.01 (0.74 – 5.47)	0.170
ab- pf+	13	9 (69.23)	7.10 (1.87 – 26.9)	0.004
ab- pf -	54	13 (24.1)	1	1

c) Association of anti-P1 antibodies and parasites with protection from clinical malaria

Group <sup>1</sup>	total number	No. with malaria <sup>2</sup> (%)	Odds ratio <sup>3</sup> (95% C.I.)	p-value
ab+ pf+	20	6 (30.0)	1.38 (0.42 – 5.97)	0.587
ab+ pf -	11	6 (54.55)	3.72 (0.98 – 14.11)	0.053
ab- pf+	35	16 (45.71)	2.53 (1.07 – 5.97)	0.035
ab- pf -	74	19 (25.68)	1	1

<sup>1</sup>Individuals were stratified according to whether they were antibody positive (scored as corrected MFI greater than the mean plus 3 standard deviations of the MFI of 20 non-exposed donors) or negative and parasite positive or negative (detected by microscopy) at cross-sectional survey.

<sup>2</sup>Malaria defined as at least one episode of fever>37.5°C plus parasitaemia>2500/μl if aged older than one year and fever >37.5°C plus any parasitaemia if aged less than one year. <sup>3</sup>Odds ratio of becoming a case of malaria obtained from a multiple logistic regression controlling for age (categorised as a factor of six months duration), location and exposure (estimated by responses to whole schizont extract)

Table 6.5 Association of antibody-parasite group with number of malaria episodes

Parasite		ab - pf -	ab - pf +	ab + pf -	ab + pf +
A4U	OR <sup>†</sup> (95% C.I.)	1	3.47 (1.34 - 9.04)	1.10 (0.42 - 2.89)	1.43 (0.55 - 3.74)
	p-value	n/a	0.011	0.837	0.469
A4 40-cycle	OR (95% C.I.)	1	5.69 (1.87 - 17.34)	2.58 (0.86 - 7.73)	1.48 (0.49 - 4.41)
	p-value	n/a	0.002	0.089	0.479
3D7	OR (95% C.I.)	1	6.93 (2.06 - 23.33)	2.21 (0.78 - 6.33)	1.59 (0.53 - 4.75)
	p-value	n/a	0.002	0.139	0.409
clinical isolate P1	OR (95% C.I.)	1	2.59 (1.09 - 6.17)	3.08 (0.88 - 10.79)	1.58 (0.44 - 5.58)
	p-value	n/a	0.031	0.079	0.477

<sup>†</sup>odds ratio of likelihood of experiencing a higher number of malaria episodes. OR calculated by ordered logistic regression controlling for age, location and exposure as estimated by response to whole schizont extract.

## Discussion

That the majority of the burden of malaria morbidity and mortality fall on young children in endemic areas must imply that significant immunity to malaria disease develops with time and exposure. Older children and adults are resistant to severe malaria and death although they remain susceptible to infection (Marsh 1992). Defining an individual as immune or non-immune however is problematic. As the risk of severe malaria disease and death declines in young children, the parasite prevalence is still rising, similarly the mean number of clinical malaria attacks falls during the period parasite prevalence is still increasing. Furthermore, during the period when young children are most at risk of severe malarial disease and death, they spend the majority of the time asymptotically parasitised (Marsh, Otoo et al. 1989). The marked differences in relationships of susceptibility to parasitisation, mild clinical disease and severe life-threatening disease and death with age, (figure 6.7), seems to that the immune mechanisms responsible for protection against severe malaria are not the same as those that protect against infection or mild disease. This is supported by modelling work suggesting that immunity to severe disease is apparent after only few symptomatic infections (Gupta, Snow et al. 1999). Although the immune effectors and their targets resulting in protection from severe disease must be the most important mechanisms to identify, in terms of vaccine design and understanding the biology of immune acquisition, it is more often that studies use mild disease

and infection as markers of susceptibility. Certainly for the studies described in this thesis, logistics of identifying large numbers of cases of severe malaria disease negated using this as our outcome. If mild malaria disease is chosen as the outcome

marker of susceptibility, then problems can arise in defining exactly what constitutes a case of clinical malaria. In some areas, 80% of children can have microscopically detectable parasites present in their blood at any one time. In this cohort of individuals 43.2 % of people sampled in a cross-sectional survey were parasitaemic. Thus the presence of parasites per se in conjunction with non-specific symptoms such as fever may not accurately define an individual's illness as being due to malaria. The studies undertaken in this thesis were embedded in a longitudinal study examining the optimal definition of clinical malaria in different age groups (Mwangi, Ross et al. 2005). The resultant definition is the definition of clinical malaria used in this work. By measuring a single putative immune response and relating it to the incidence of clinical malaria over time, as was done in these studies, it must be assumed that this response, if present, can be measured in every individual, at a single point in time. The results outlined in chapter 3, and previous work from this area, suggest that this may not be entirely the case (Bull, Lowe et al. 2002; Kinyanjui, Mwangi et al. 2004; Polley, Mwangi et al. 2004). It was noted that the prevalence and amount of antibodies to a range of blood-stage antigens were consistently higher amongst children asymptomatically parasitised compared to those with no parasites detectable by microscopy. Given that all children in these areas move between being parasite positive and parasite negative relatively frequently, it may be that immune responses measured are short-lived and as a result the ability to detect the response short-lived.

**Table 6.6 Association of serum IgG levels to recombinant domains of PfEMP1 in October 2000 with clinical malaria in Chonyi, Kenya for the period October 2000 until March 2001**

<i>Chonyi (age adjusted)</i>		<i>slide all</i>	<i>n=596</i>	
Antigen	Odds Ratio	p-value	[95% Conf.	Interval]
DBL1 $\alpha$	0.75	0.580	0.26	2.11
DBL2 $\beta$	0.63	0.472	0.18	2.21
CIDR1 $\alpha$	0.71	0.365	0.33	1.50
DBL4 $\gamma$	0.89	0.791	0.37	2.13
DBL5 $\beta$	0.72	0.517	0.26	1.95
<i>Chonyi (age adjusted)</i>		<i>slide positive</i>	<i>n=197</i>	
Antigen	Odds Ratio	p-value	[95% Conf.	Interval]
DBL1 $\alpha$	1.79	0.423	0.43	7.49
DBL2 $\beta$	0.51	0.491	0.07	3.51
CIDR1 $\alpha$	0.51	0.287	0.15	1.76
DBL4 $\gamma$	2.47	0.251	0.53	11.64
DBL5 $\beta$	0.66	0.691	0.09	4.88
<i>Chonyi (age adjusted)</i>		<i>slide negative</i>	<i>n=399</i>	
Antigen	Odds Ratio	p-value	[95% Conf.	Interval]
<b>DBL1<math>\alpha</math></b>	<b>0.05</b>	<b>0.020</b>	<b>0.04</b>	<b>0.63</b>
DBL2 $\beta$	0.26	0.208	0.32	2.10
CIDR1 $\alpha$	0.72	0.553	0.25	2.10
DBL4 $\gamma$	0.49	0.290	0.14	1.81
DBL5 $\beta$	0.49	0.343	0.11	2.11

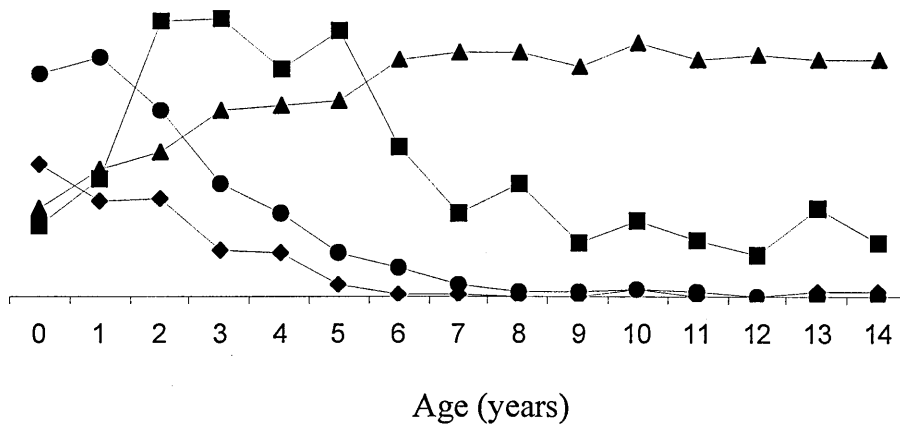
Odds Ratios are adjusted for age and reactivity to schizont extract.

**Table 6.7 Association of serum IgG levels to recombinant domains of PfEMP1 in October 2000 with clinical malaria in Ngerenya, Kenya for the period October 2000 until March 2001**

<i>Ngerenya (age adjusted)</i>		<i>slide all</i>			<i>n=617</i>
Antigen	Odds Ratio	p-value	[95% Conf.	Interval]	
DBL1 $\alpha$	1.04	0.925	0.47	2.32	
DBL2 $\beta$	1.32	0.621	0.44	3.94	
CIDR1 $\alpha$	1.15	0.620	0.65	2.07	
DBL4 $\gamma$	0.64	0.333	0.26	1.58	
DBL5 $\beta$	0.84	0.672	0.37	1.89	
<i>Ngerenya (age adjusted)</i>		<i>slide positive</i>			<i>n=148</i>
Antigen	Odds Ratio	p-value	[95% Conf.	Interval]	
DBL1 $\alpha$	1.76	0.90	0.51	6.10	
DBL2 $\beta$	1.51	0.749	0.12	18.77	
CIDR1 $\alpha$	2.20	0.220	0.622	7.81	
DBL4 $\gamma$	0.85	0.864	0.15	4.93	
DBL5 $\beta$	0.97	0.977	0.18	5.33	
<i>Ngerenya (age adjusted)</i>		<i>slide negative</i>			<i>n=469</i>
Antigen	Odds Ratio	p-value	[95% Conf.	Interval]	
DBL1 $\alpha$	0.96	0.944	0.31	2.97	
DBL2 $\beta$	1.12	0.861	0.31	4.11	
CIDR1 $\alpha$	0.79	0.549	0.38	1.66	
DBL4 $\gamma$	0.57	0.314	0.19	1.69	
DBL5 $\beta$	0.76	0.589	0.28	2.05	

Odds Ratios are adjusted for age and reactivity to schizont extract.

Those parasite negative and antibody negative may be either unable to make the response and so a true antibody negative, or may be perfectly capable of making such a response if challenged and thus falsely classified as antibody negative. The importance of these potentially short-lived responses have been highlighted recently in a number of studies where no relationship between a given immune response and protection was observed in the cohort as a whole, but when individuals parasite positive at the time of sampling were analysed separately a clear protective effect was observed (Bull, Lowe et al. 2002; Kinyanjui, Mwangi et al. 2004; Polley, Mwangi et al. 2004). The 'boosting' of antibody responses amongst those parasite positive at the time of sampling seen in these studies and others is not as apparently straightforward as at first thought. When the relationship between antibody levels and parasites was analysed by future disease experience, ie individuals stratified by those who became a case of clinical malaria in the subsequent six months and those who did not, the increased intensity and prevalence of antibody responses in association with parasites was only apparent in those who did not go on to become a case of clinical malaria (table 6.1). It is difficult to assess whether this fact is telling something important about anti-malaria immunity.



**Figure 6.7**

Relative risks of infection (triangles), morbidity (squares), severe disease (circles) and death (diamonds) among a population aged 0-15 years located in a stable endemic area on the Kenyan coast.

(Adapted from (Snow and Gilles 2002))



It may be that the ability to respond appropriately to challenge in the form of parasites, by the production of relatively cross-reactive antibodies or a broader range of antibody responses, or even the production of antibodies targeting less immunogenic conserved targets is a marker of relative immunity and those individuals unable to respond in this way are more susceptible. Or it may simply be that both parasite positivity and antibody positivity are surrogate markers of cumulative exposure and that there is no direct association between the two variables. Stringent measures were used to ensure that differences in previous exposure between the two groups were accounted for. Within the multiple logistic models, age, a proxy measure of exposure (responses to whole schizont extract) and location (high or low transmission) were all included as significant co-dependent variables. Nonetheless it is impossible to exclude the possibility that previous exposure remained inadequately accounted for. Certainly previous work has demonstrated a similar finding and in this work no measure of previous exposure altered the association between antibodies and parasites (Bull, Lowe et al. 2002).

The importance of the relationship between asymptomatic parasitisation and antibody responses on the one hand, and protection from or susceptibility to subsequent disease on the other was given further support in this work with the identification of a susceptible sub-group of individuals. By stratifying individuals according to their antibody and parasite status at cross-sectional survey, we were able to identify those children most at risk of subsequent clinical malaria. Those children with parasites but with no concomitant rise in heterologous antibody levels experienced significantly more attacks of clinical malaria in the subsequent six months even after attempting to control for confounding variables such as age and

exposure (table 6.3, table 6.4 and figure 6.5). This susceptibility was the case with responses against any of the isolates tested. It has been hypothesised that the development of immunity against clinical malaria is associated with the ability to maintain chronic asymptomatic infections. Chronic asymptomatic infections themselves have been speculated to directly protect against super-infections, a process called premunition (Serjent and Parrot 1935). Work in humans suggested that premunition was related to the multiplicity of clones present within the established controlled infection (al-Yaman, Genton et al. 1997; Smith, Felger et al. 1999). The protection afforded by the ability to maintain chronic infections is supported by the observation that, with age, disease incidence decreases at a time when the prevalence of parasitaemia increases (Marsh 1992). This is of course at odds with the observation in this chapter that being parasitaemic *per se* was associated with an increased susceptibility to mild clinical malaria. Previous work from this area showed no association between asymptomatic parasite carriage at the end of a dry season and either protection from or susceptibility to severe malarial disease (Bull, Lowe et al. 2002). Work carried out in Senegal under low to moderate transmission (EIR 5-10) demonstrated an association between asymptomatic parasitisation at the end of a dry season with protection against subsequent mild clinical malaria (Males, S *published abstract*). It is not clear if these contrasting results reflect different methodologies, different outcome measures of clinical malaria or are due to different levels of exposure amongst the individuals studied. The increased susceptibility observed in those with parasites and poor concomitant heterologous antibody responses might reflect a poor inherent ability to respond appropriately. Such children have been reported in a number of studies (Giha, Staalsoe et al. 1999; Ofori, Dodoo et al. 2002; Kinyanjui, Bull et al. 2003) although

on an individual basis it is not known if these children are actually more susceptible to clinical malaria. There was also a suggestion within this data, and previous data from the same area, that having antibodies to the infected erythrocyte surface in the absence of detectable parasites was associated with susceptibility (Kinyanjui, Mwangi et al. 2004). This may simply indicate a recently treated symptomatic infection and may thus be reflective of a sub-population of individuals at increased risk in terms of either challenge or inherent host susceptibility factors.

Of course all of these differences in susceptibility may simply be highlighting differences in exposure and thus risk between the groups. Those with parasites at the time of sampling have a defined exposure, whereas those without microscopically detectable parasites will be a more heterogeneous group in terms of likelihood of risk and thus any difference in protection or susceptibility may be more difficult to disentangle.

Studies identifying targets for protective immunity on the infected erythrocyte surface have had conflicting results. Whereas following infection children develop antibodies specific to the infecting isolate associated with protection (Bull, Lowe et al. 1998), it is not clear how relatively cross-reactive antibodies, or responses directed against less immunogenic conserved targets are involved in protective immunity. In Kenya, the ability of sera to agglutinate a randomly selected isolate was not associated with protection (Bull, Lowe et al. 1998; Bull, Lowe et al. 2002). However in Sudan, antibodies directed against an isolate from Ghana were associated with protection while antibodies to six Sudanese isolates were not and in Ghana responses to a Sudanese isolate and to a Ghanaian isolate demonstrated an

association with protection but responses to another Ghanaian isolate did not (Giha, Staalsoe et al. 2000; Dodoo, Staalsoe et al. 2001). It should be noted however that in none of these studies was asymptomatic parasitaemia taken into account at the time the sera were assayed. Whether antibodies directed against these isolates are genuinely more protective or these isolates are in some way different in the antigens expressed, perhaps expressing to a greater degree a cross-reactive or conserved subset of variant antigens or whether these results are simply reflecting differences in prevalence and the ability of these measured responses to act as surrogate markers of protection is unknown. In this study, responses to four parasite lines, including one selected to express one dominant *var* gene, were associated with protection in those with asymptomatic parasitaemia. Identifying the targets for this response is of considerable importance both for vaccine development and improved understanding of the acquisition of immunity in children in malaria endemic areas. It was with this aim we assessed whether antibodies directed against recombinant domains of A4 *PfEMP1* were also associated with protection from clinical malaria.

Overall in each area studied, we could find no association with the presence of domain-specific antibodies and protection (table 6.6 and table 6.7). However by categorising individuals according to the presence or absence of parasites at cross-sectional survey, we found an association with disease incidence in those with anti-DBL1 $\alpha$  antibodies and no detectable parasites. This association was only evident in the higher transmission area. It is difficult to satisfactorily explain this result. Certainly previous studies examining the association between circulating antigen-specific antibodies and protection from clinical malaria the protective effect was only seen in those individuals possessing both antibodies and parasites at the time of

bleed (Kinyanjui, Mwangi et al. 2004; Polley, Mwangi et al. 2004). It is likely that by expressing each domain as a recombinant fusion protein, important conformational epitopes are lost and also possible that targets on each recombinant protein are functionally unimportant *in vivo*.

# Chapter 7

## Concluding Remarks

The results presented in this thesis have highlighted the complicated and effective acquisition of natural immunity that occurs in malaria endemic areas. By examining responses to the surface of the infected red cell, from individuals in areas of differing transmission intensity it was possible to compare the effects of different levels of challenge in attaining specific anti-red cell surface immune responses and the effect these responses had on the likelihood of suffering clinical malaria.

In particular it was striking that children resident in both the high and the low transmission area develop such a wide breadth of responses. This was particularly apparent with responses detected to the laboratory cloned isolate A4U. This parasite had been selected to display one variant of *PfEMP1* on the surface of infected erythrocytes through use of a monoclonal antibody, BC6. Despite this 100% of children whose immune responses were examined showed evidence of recognition of this variant by the age of 7 years in the high transmission area and 10 years in the low transmission area. The broad extent of responses present within this population of children was further illustrated by the increased prevalence and intensity of recognition of this laboratory clone in the presence of asymptomatic parasite carriage. That these responses were almost totally absent amongst children from the same area with symptomatic malaria, both at the time of disease presentation and during 12 weeks of convalescence may imply that the ability to maintain asymptomatic parasite carriage is not only associated with a broader range of responses but also

possibly that susceptibility to clinical malaria may be associated with the breadth of immune response. Certainly the 'boosting' seen in both antibody prevalence and intensity in the presence of asymptomatic parasite carriage was only apparent amongst those children who remained protected from clinical malaria during the season immediately following the cross-sectional bleed. A result which must tell us something important regarding an individual's ability to remain protected from clinical disease.

Regarding the effects of host genotype on the ability of an individual to produce a specific immune response, results in this thesis support earlier work where a significantly higher titre of anti-infected erythrocyte antibodies was noted in children with HbAS compared to HbAA (Marsh, Otoo et al. 1989), and more recent work whereby the presence of HbAS genotype was associated with enhanced recognition of two heterologous clinical isolates among Gabonese children (Cabrera, Cot et al. 2005). There was no effect of thalassaemia status on immune responsiveness with regards recognition of the same isolates.

There remains no reliable and reproducible *in vitro* marker of protection from clinical malaria. Even defining an individual child as immune or non-immune is problematic as has been discussed. Within the cohort of individuals studied in this work, the presence of an asymptomatic parasitaemia at the end of a low transmission season conferred increased risk of subsequent clinical malaria. What was surprising was that this increased susceptibility was almost entirely accounted for by those individuals with parasites but with no concurrent detectable antibody response to the

surface of *P. falciparum* infected erythrocytes. This association was not variant specific, being present with all 4 parasite isolates tested although the level of protection afforded by antibodies present with asymptomatic parasite carriage did differ between the isolates tested.

The question of what might be the target of these responses is a question of considerable importance and interest. It was unexpected and somewhat exciting to demonstrate the remarkably strong correlation in individual responses between the different parasite clones and isolates studied. That, statistically, this could not be accounted for simply by exposure was even more unanticipated. Although not absolutely certain, the most likely erythrocyte surface exposed antigen as a target for these responses remains *PfEMP1* and models akin to those hypothesised for placental malaria by Beeson and colleagues can be applied in this case (Beeson, Rogerson et al. 2006). It may be that within different *PfEMP1* variants, each highly diverse, there exists more conserved perhaps less immunogenic regions. Antibodies to these conserved regions may be important in conferring protection. Alternatively there may be a restricted number of nonetheless antigenically diverse *PfEMP1* variants and acquiring a repertoire of antibodies against these would confer protection. That we were unable to see any association between recognition of individual A4 *PfEMP1* domains and protection from clinical disease was possibly due to the method of expression chosen, with conformational epitopes being of greater importance, or perhaps the regions chosen for expression were too long and thus lacked specificity for any potential protective response to be recognised as such. Of course the possibility of the presence of an as yet unidentified surface-expressed antigen remains. The identification and characterisation of protective targets on the



infected erythrocyte surface is of profound importance in the understanding of the naturally acquired protective immune response and in the development of an effective malaria vaccine.

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